UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA
Departamento de Bioquímica y Biología Molecular III

TESIS DOCTORAL

Modulación de la respuesta inflamatoria secundaria a la isquemia/reperfusión pulmonar en un modelo experimental de cirugía de recesión pulmonar en cerdos

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Lisa Rancan

Directores

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Ignacio Garutti Martínez

Madrid, 2015

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Memoria presentada por

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para la obtención del grado de Doctor

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Elena Vara, Catedrática de Bioquímica y Biología Molecular, Carlos María Simón Adiego, Profesor Asociado y FEA del Departamento de Cirugía (Servicio de Cirugía Torácica del HGUGM), Facultad de Medicina de la UCM e Ignacio Garutti, FEA del Servicio de Anestesiología del HGUGM,

CERTIFICAN:

Que el trabajo titulado **"Modulación de la respuesta inflamatoria secundaria a la isquemia/reperfusión pulmonar en un modelo experimental de cirugía de resección pulmonar en cerdos"** del que es autora Dñª. Lisa Rancan, ha sido realizado bajo nuestra dirección.

Lisa ha participado activamente con nosotros como investigadora en diferentes proyectos de investigación subvencionados (PI10/01900, PI10/00986, AP101072012, (PI13/00700, FMM14/08) enfocados en la posible modulación de la respuesta inflamatoria secundaria a la cirugía de resección pulmonar mediante diferentes estrategias de precondicionamiento.

Lisa ha participado activamente tanto en los procesos quirúrgicos como anestésicos y ha sido la responsable de todas las determinaciones bioquímicas. La participación en estos proyectos le ha permitido realizar una serie de actividades experimentales, conducentes a la obtención del Grado Académico de Doctor. En este período ha obtenido una serie de logros y resultados que se han materializado hasta la fecha en 7 publicaciones en revistas indexadas en el Journal of Citation Reports, varias de ellas como primer autor. También ha participado en diversos congresos científicos tanto nacionales como internacionales, donde se han presentado los resultados de su trabajo. Por todo ello, consideramos que el trabajo realizado reúne las características necesarias de originalidad, planteamiento adecuado de la hipótesis de trabajo, desarrollo metodológico adecuado y discusión critica de los resultados para ser defendida como tesis doctoral.

Madrid, a 10 de Abril de 2015

Fdo.: E. Vara **C. Simón** **I. Garutti**

A Chema,

Por ser, por estar.

Alla mia famiglia,

Stella polare in ogni mio viaggio.

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"Si possono varcare monti, navigare mari, andare alla ricerca di bellezze uniche ed incontrare meraviglie rare, toccare le stelle e contemplarle, ma che senso avrebbe ciò se non si avesse nessuno a cui raccontarlo?"

- *Cicerone*

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INDICE

ARTICULÓS QUE CONSTITUYEN LA TESIS

Esta tesis se basa en los siguientes artículos a los que se refiere a lo largo del texto utilizando los números romanos:

I. L. Huerta, L. Rancan, C. Simón, J. Isea, E. Vidaurre, E.Vara, I. Garutti and F. González-Aragoneses. Ischaemic preconditioning prevents the liver inflammatory response to lung ischaemia/reperfusion in a swine lung autotransplant model. Eur J Cardiothorac Surg. 2013 Jun;43(6):1194-201.

II. L. Rancan, L. Huerta, G. Cusati, I. Erquicia, J. Isea, S.D. Paredes, C. García, I. Garutti, C. Simón, E. Vara. Sevoflurane prevents liver inflammatory response induced by lung ischemia-reperfusion. Transplantation. 2014 Dec 15;98(11):1151-7.

III. I. Garutti*, L. Rancan*, C. Simón, G. Cusati, G. Sanchez-Pedrosa, F. Moraga, L. Olmedilla, M.T. Lopez-Gil, E. Vara. Intravenous lidocaine decreases tumor necrosis factor alpha expression both locally and systemically in pigs undergoing lung resection surgery. Anesth Analg. 2014 Oct;119(4):815-28. * Ambos autores han contribuido igualmente

IV. L. Rancan*, C. Simón*, E. Marchal-Duval, J. Casanova, S.D. Paredes, A. Calvo, C. García, D. Rincón, I. Garutti, E.Vara. Lidocaine administration controls microRNAs alterations observed after lung ischemia reperfusion injury. Anesthesiology (en revisión). * Ambos autores han contribuido igualmente

V. L. Rancan, G. Sánchez-Pedrosa, K. Aymonnier, J. Casanova, C. Muñoz, D. Rincón, C.Simón, I. Garutti, E. Vara. Glycocalyx degradation after pulmonary resection surgery. British Journal of Anaesthesia (en revision).

VI. L. Rancan, D. Rincón, L. Huerta, S.D. Paredes, C. García, I. Garutti, F. González-Aragoneses, C. Simón, E. Vara. Chemokine involvement in lung injury secondary to ischaemia/reperfusion. World journal of surgery (enviado).

VII. F.de la Gala, P. Piñeiro; I. Garutti; A. Reyes, L. Olmelilla, P. Cruz, P. Duque, J. Casanova, L. Rancan, P. Benito, E. Vara. Systemic and alveolar inflammatory response in the dependent and nondependent lung in patients undergoing lung resection surgery. European Journal of Anesthesiology, en prensa.

SUMMARY

"No recibimos sabiduría, debemos descubrirla nosotros mismos; tras un largo viaje que nadie puede librarnos o hacer por nosotros".

- *Marcel Proust*

Abstract

Background: Lung resection surgeries (LRS) cause an inflammatory response that triggers the ischemia reperfusion injury (IRI) associated with local and systemic alterations. Different strategies have been investigated to prevent the IRI. Among them, preconditioning has emerged as a promising strategy. Also lidocaine, a local anaesthetic with anti-inflammatory activity seems promising but its modulation of IRI has not been investigated yet. The general aim of this study was to investigate a possible role of the proinflammatory and proapoptotic mediators in the lung IRI and to determinate whether these alterations can be accompanied by glycocalyx and miRNAs expression alterations. In addition, a possible modulation of this injury by preconditioning techniques and lidocaine administration was investigated.

Methods: Large White pigs underwent LRS. In addition, some animals received ischemic or anaesthetic (sevoflurane) preconditioning and others received lidocaine. Broncho alveolar lavage, plasma, lung and liver samples were taken before pneumonectomy, before reperfusion and at two time-points after reperfusion in order to perform biochemical determinations.

Results: LRS caused lung damage with increased inflammation apoptosis. These alterations were also evident in systemic and hepatic samples. Increased miRNAs expression and glycocalyx degradation were also observed after reperfusion. Both preconditioning and lidocaine administration reduced significantly the abovementioned alterations.

Conclusions: Our results suggest that both preconditioning and lidocaine can prevent LRS-induced lung IRI through reduced expression of inflammation and apoptosis. In addition, lidocaine reduced significantly the alterations of miRNAs and glycocalyx caused by IRI.

Introduction

Several pathophysiological mechanisms have been described to explain the lung injury that occurs during LRS. It seems that inflammation plays a pivotal role increasing oxidative stress and apoptosis and causing barrier disruption (endothelial and epithelial). The consequent injury is called ischemia reperfusion injury (IRI). During IRI, endothelial cells

suffer swelling and detachment from the basement membrane (Kurose et al. 1997) which suggests an involvement of the endothelial glycocalyx. On the other hand, microRNAs (miRNAs), short RNA sequences that act as post-transcriptional regulators, have emerged as promising disease biomarkers. In fact, recent studies have examined the possibility that changes in miRNAs expression could be used as biomarkers for IRI (Shapiro et al. 2011) and transplants (Zhang et al. 2013).

Is has been observed that lung IRI induces damage to remote organs too (Esme et al. 2006). Liver seems to be particularly sensitive to IRI when it is directly (Serracino-Inglott et al. 2001) or indirectly (Kelly 2003) involved. Different methods have been developed to mitigate IRI. Remote ischaemic preconditioning (repetitive ischaemic episodes in an organ that is remote from the organ to be protected), has been proposed to prevent the damage caused by cardiac, hepatic or brain ischaemia. Also, it has been proposed the anaesthetic preconditioning with volatile anaesthetics (sevoflurane, halothane and isoflurane, among others) which exhibit anti-inflammatory effects in different organs and systems (Yonemochi et al. 2006, Tanaka et al. 2004b, Xiong et al. 2003, Plachinta et al. 2003). Anti-inflammatory effects have been related also with another anaesthetic, the lidocaine (Hollmann et al. 2001, McCarthy et al. 2010, Picardi et al. 2013) that also acts on endothelial lung cells attenuating the damage to these cells caused by cytokines (Lan et al. 2005). Therefore, we hypothesized that IRI caused during LRS causes an inflammatory response that also affects distant organs due to the release of proinflammatory and apoptotic mediators into the bloodstream and that it is possible to modulate this inflammatory response through preconditioning techniques: ischemic or anaesthetic.

Materials and Methods

For the content of this section, we refer to the papers that constitute this thesis

Results

i. Lung resection surgery induced a significant increase in the levels of inflammatory and apoptotic mediators, both locally and systemically.
ii. These alterations may be secondary to the I/R per se or the OLV required in these surgical procedures
iii. The increased levels of proinflammatory mediators was accompanied by changes in the expression profile of microRNAs and alterations in the integrity of glycocálix.

iv. Anaesthetic administration of sevoflurane or lidocaine significantly diminished the abovementioned alterations both locally and systemically.

Discussion

It seems that one of the main alterations due to I/R consists in increased inflammatory response both local and systemic. In accordance with that, in our studies we have observed alterations of the inflammatory response, both locally and systemically, in all the analysed models. Locally, we observed that the ischemia *per se* induces alterations of pro-inflammatory and anti-inflammatory cytokines expression. In addition, we observed that, after reperfusion, these alterations were even greater which is in accordance with previous studies from our group (Simon Adiego et al. 2011, Casanova et al. 2011), as well as with others (de Perrot et al. 2003). These alterations were also evident in systemic and hepatic samples.

In our studies, lidocaine administration reduced the abovementioned alterations of the inflammatory and apoptotic responses. Preconditioning, both ischemic and anaesthetic, results effective too in the modulation of the IRI. In particular, we observed that it was effective also at systemic and hepatic levels, reducing the inflammatory response and the apoptosis.

All the previously described alterations contribute to develop a pulmonary oedema. In our studies, the oedema was evident in all the experimental models and all the tested strategies of damage modulation reduced it significantly.

Recently it has been proposed that the oedema could be caused not only by the inflammatory response, but also by the loss of integrity of the endothelial glycocalyx (Rehm et al. 2007). In accordance with this hypothesis, we observed a degradation of glycocalyx after reperfusion. Also, this degradation was accompanied, in our models, by an increased neutrophils activation. Lidocaine administration reduced significantly the glycocalyx degradation and the expression of adhesion molecules.

Several miRNAs have been related to pulmonary diseases associated to inflammatory process (Xie et al. 2012) as well as after IRI (Weiss et al. 2012). In accordance with these studies, we observed significant alterations of the expression of several miRNAs after reperfusion.

Resumen

Antecedentes: La cirugía de resección pulmonar (CRP) causa una respuesta inflamatoria que desencadena un daño por isquemia-reperfusión (IRI) lo cual se asocia con alteraciones locales y sistémicas. Diferentes estrategias han sido investigadas para reducir el IRI. Entre ellas, el precondicionamiento parece una estrategia prometedora. También la lidocaína, un anestésico local que posee capacidad anti-inflamatoria parece prometedor. El objetivo general de este trabajo, fue investigar el posible papel de los mediadores proinflamatorios y apoptóticos en el daño secundario a la isquemia-reperfusión pulmonar y determinar si estos cambios van acompañados por modificaciones en alteraciones del glicocálix y/o la expresión de miRNAs. Además se investigó la posible modulación de este daño mediante técnicas de precondicionamiento o la administración de lidocaína

Métodos: Cerdos de raza Large White fueron sometidos a CRP. Para la protección pulmonar se emplearon técnicas de precondicionamiento, isquémico o anestésico (sevoflurano) y administración de lidocaína. Muestras de lavado bronco-alveolar, plasma, pulmón e hígado fueron tomadas antes de la neumonectomía, antes de la reperfusión y en dos tiempos después de la reperfusión con el fin de realizar las determinaciones bioquímicas.

Resultados: La cirugía causó un daño pulmonar con aumento de inflamación y apoptosis. Estas alteraciones se observaron también a nivel sistémico y hepático. También se observó aumento de la expresión de miRNAs y una degradación del glicocálix después de la reperfusión. Tanto el precondicionamiento, quirúrgico o anestésico, como la administración de lidocaína redujeron significativamente estas alteraciones.

Conclusiones: Nuestros resultados sugieren que tanto el precondicionamiento, quirúrgico o anestésico, como la lidocaína pueden prevenir el IRI inducido por las CRP reduciendo la inflamación y el apoptosis. Además, la lidocaína reduce significativamente las alteraciones observadas a cargo de miRNAs y glicocálix.

Introducción

Varios mecanismos fisiopatológicos se han descrito para explicar la lesión pulmonar que se produce tras las CRP. Parece que la inflamación juega un papel fundamental contribuyendo a un aumento de estrés oxidativo y apoptosis y produciendo alteración de las barreras endotelial y epitelial. El consecuente daño se denomina lesión por isquemia-reperfusión (IRI). Durante el IRI se observa hinchazón y desprendimiento de la membrana basal por parte de las células endoteliales (Kurose et al. 1997) lo cual sugiere una implicación del glicocálix endotelial. Por otro lado, los microRNAs (miRNAs), secuencias cortas de ARN que actúan como reguladores post-transcripcionales, han surgido como prometedores marcadores de diferentes enfermedades. De hecho, estudios recientes han examinado la posibilidad de que cambios en la expresión de los miRNAs podrían utilizarse como marcadores para el IRI (Shapiro et al. 2011) y los trasplantes (Zhang et al. 2013).

Se ha observado que el IRI pulmonar induce también una respuesta sistémica que puede dañar órganos lejanos (Esme et al. 2006). El hígado parece ser particularmente sensible al IRI tanto cuando es implicado directamente (Serracino-Inglott et al. 2001) como indirectamente (Kelly 2003). Diferentes métodos han sido desarrollados para mitigar el IRI. El precondicionamiento isquémico remoto (episodios isquémicos repetitivos en un órgano que es remoto respecto al órgano a proteger) se ha propuesto para reducir el daño causado por isquemias a nivel cardiaco, hepático o cerebral. También se ha propuesto el precondicionamiento con anestésicos volátiles (sevoflurano, halotano e isofluorano, entre otros) que presentan efectos anti-inflamatorios en diferentes órganos y sistemas (Yonemochi et al. 2006, Tanaka et al. 2004b, Xiong et al. 2003, Plachinta et al. 2003). Efectos antiinflamatorios se han relacionado también con otro anestésico, la lidocaína (Hollmann et al. 2001, McCarthy et al. 2010, Picardi et al. 2013), la cual además actúa sobre las células endoteliales pulmonares atenuando el daño a su cargo producido por las citoquinas (Lan et al. 2005). Por lo tanto, en este trabajo se ha hipotetizado que el IRI producido por las CRP no sólo afecta al funcionamiento del órgano involucrado en la cirugía, sino que también produce una respuesta inflamatoria sistémica debido a la liberación de mediadores proinflamatorios y apoptóticos en el torrente sanguíneo y que esto se puede modular a través de diferentes técnicas.

Materiales y Métodos

Para los materiales y métodos se hace referencia a los artículos que forman parte de esta tesis doctoral.

Resultados

i. La cirugía de resección pulmonar indujo un aumento significativo de los niveles de mediadores inflamatorios y/o apoptóticos, tanto a nivel local como sistémico
ii. Estos cambios podrían ser secundarios tanto a la respuesta inflamatoria quirúrgica así como al daño pulmonar producido durante la ventilación unipulmonar (OLV) técnica ampliamente utilizada durante las intervenciones torácicas
iii. El aumento de los niveles de mediadores proinflamatorios fue acompañado por modificaciones en el perfil de expresión de microRNAs y alteraciones en la integridad del glicocálix
iv. La modulación anestésica con sevoflurano o lidocaína, disminuyó significativamente estos efectos tanto a nivel local como sistémico

Discusión

Una de las principales alteraciones producidas por el IRI parece ser un aumento de la respuesta inflamatoria local y sistémica. De acuerdo con eso, en nuestros estudios hemos observado alteraciones de la respuesta inflamatoria, tanto a nivel local como sistémico, en todos los modelos analizados. A nivel local, se observó que la isquemia *per se* induce alteraciones de la expresión de citoquinas pro-inflamatorias y anti-inflamatorias. Además, se observó que, después de la reperfusión, estas alteraciones eran aún mayores, lo cual está de acuerdo con estudios previos de nuestro grupo de (Simon Adiego et al. 2011, Casanova et al. 2011) y de otros (de Perrot et al. 2003). Alteraciones inflamatorias también se observaron a nivel sistémico y hepático.

En nuestros estudios, la administración de lidocaína redujo las alteraciones de la respuesta inflamatoria y apoptótica mencionados anteriormente. El precondicionamiento, tanto isquémico como anestésico, resultó también eficaz en la modulación del IRI. En particular, se observó su eficacia a nivel sistémico y hepático donde redujo tanto la respuesta inflamatoria como la apoptótica.

Todas las alteraciones descritas anteriormente contribuyen a desarrollar un edema pulmonar. En nuestros estudios, el edema era evidente en todos los modelos analizados y todas las estrategias de modulación del daño investigadas lo redujeron significativamente.

Recientemente se ha propuesto que el edema podría ser causado no sólo por la respuesta inflamatoria, sino también por la pérdida de la integridad de la glicocálix endotelial (Rehm et al. 2007). De acuerdo con esta hipótesis, se observó una degradación del glicocálix después de la reperfusión. Además, esta degradación era acompañada, en nuestros modelos, por un aumento de la activación de los neutrófilos. La administración de lidocaína redujo significativamente la degradación del glicocálix y la expresión de las moléculas de adhesión.

Varios miRNAs se han relacionado con enfermedades pulmonares asociadas a procesos inflamatorios (Xie et al. 2012) así como después del IRI (Weiss et al. 2012). De acuerdo con estos estudios, se observó una alteración importante de la expresión de varios miRNAs después de la reperfusión.

INTRODUCCIÓN

"No recibimos sabiduría, debemos descubrirla nosotros mismos; tras un largo viaje que nadie puede librarnos o hacer por nosotros".

- *Marcel Proust*

Lesión por isquemia/reperfusión

La isquemia/reperfusión es un proceso relativamente frecuente en diferentes situaciones clínicas, incluyendo cirugía y trasplante. La fisiopatología de este proceso incluye no solo el fallo del aporte de sangre en un momento determinado, sino también el efecto posterior de la reperfusión sobre las células isquémicas, con un metabolismo disminuido, reduciendo su capacidad de detoxificar los radicales libres generados en la respiración celular y/o otros procesos metabólicos. El daño celular activa una respuesta inflamatoria con infiltración de polimorfonucleares a los tejidos, los cuales a su vez pueden producir radicales libres de oxígeno, aumentando así el daño isquémico. La acumulación de mediadores proinflamatorios tales como citoquinas, metabolitos del araquidónico y/o el complemento, endotoxinas procedentes de células necróticas etc. justificarían en parte el fallo multiorgánico relacionado con la I/R en algunos pacientes.

Se puede definir la isquemia, como la limitación del flujo sanguíneo; es una condición en la cual un tejido es privado de oxígeno y nutrientes. Esto induce alteraciones de los fenómenos respiratorios celulares y del metabolismo celular del órgano afectado que pasa de aerobio a anaerobio con disminución del nivel energético. Hay una creciente evidencia de que el daño originado a los tejidos ocurre no solo durante la fase hipóxica o isquémica, sino también durante la re-oxigenación del tejido (Wilkins et al. 1994, Shinozawa, Koike 2003).

Es generalmente aceptado que los radicales libres juegan un papel central en el daño por reperfusión. Cuando las células son privadas de oxígeno, deben depender de la glicolisis

anaeróbica y de los depósitos de glucógeno para la síntesis de ATP. Se acumula NADH y lactato, y todos los componentes del sistema de transporte de electrones mitocondrial están saturados puesto que no pueden transferirse electrones al oxígeno. El potencial de membrana mitocondrial está aumentado y cuando se reintroduce el oxígeno se producen rápidamente grandes cantidades de especies reactivas de oxígeno (ROS), superando los mecanismos de captura.

Las ROS fluyen a través de la célula, dañando los lípidos de la membrana, el DNA y otros constituyentes celulares vitales, lo que conduce a la muerte celular. Este proceso que se caracteriza por un aumento de la permeabilidad microvascular, edema y necrosis tisular, se asocia generalmente con la formación de radicales libres y la infiltración de neutrófilos (Grace 1994, de Perrot et al. 2003) que pueden contribuir al daño celular a través de diferentes mecanismos: (i) Liberación de radicales libres (O2−; OH−) en el burst respiratorio de la NADPH oxidasa, (ii) liberación de enzimas proteolíticos (elastasa, catepsina G y proteinasa), (iii) estimulación de la liberación de citoquinas por las células locales, promoviendo así una mayor infiltración de neutrófilos lo que a su vez contribuiría a la disminución del flujo sanguíneo (de Perrot et al. 2003, Vermeiren et al. 2000, King et al. 2000, Ambrosio, Tritto 1999).

En los tejidos privados de oxígeno (isquemia), el fallo de la cadena de transporte de electrones conduce a la disminución de los niveles de ATP (Jennings et al. 1983). Además el metabolismo anaeróbico induce un aumento de la producción de ácido láctico; esta acidosis altera la homeostasis celular: hay una pérdida de Ca^{2+} y una alteración de los gradientes iónicos, con la subsecuente pérdida de enzimas esenciales y proteínas (Chaudry et al. 1981). En adición, el AMP se convertirá en adenosina, xantina e hipoxantina (Van Bilsen et al. 1989), sustratos de la xantina oxidasa. Este enzima funciona normalmente como xantina deshidrogenasa pero durante la isquemia funciona en sentido contrario y

parece jugar un papel fundamental en la producción de radicales libres durante el daño inducido por la isquemia-reperfusión (I/R) (Chambers et al. 1985, McCord 1985). Las consecuencias de este daño incluyen pérdida de Ca^{2+}, edema, daño secundario a los radicales libres y muerte celular (Reimer et al. 1983).

La I/R se asocia con la producción del radical superóxido (O_2^-) generado por los sistemas NADPH oxidasa y xantina oxidasa. En presencia de la superóxido dismutasa (SOD), el O_2^- es convertido en peróxido de hidrógeno (H_2O_2) y se forma el radical hidroxilo en la reacción de Harber– Weiss (Fenton). Además, el O_2^- puede interaccionar con el óxido nítrico (NO) originando peroxinitrito ($ONOO^-$), un oxidante que rápidamente nitrosila residuos de tirosina de las proteínas celulares (Beckman, Koppenol 1996). El NO tiene una acción controvertida en la producción del daño por reperfusión (Nonami 1997). Por un lado, su ausencia se ha relacionado con una mayor adhesión de los neutrófilos al endotelio vascular y condiciona un incremento en el tono vascular. Así, las sustancias donadoras de NO han mostrado un efecto cardioprotector durante la reperfusión. Sin embargo, también existe evidencia de que el NO es capaz de condicionar daño tisular durante la reperfusión por varios mecanismos, incluida la formación de radicales libres (Schulz, Wambolt 1995). El papel exacto del NO en la producción del daño no ha sido totalmente aclarado. Lefer et al. demostraron que el $ONOO^-$ a concentración nanomolar inhibe la interacción al endotelio de los leucocitos y protege frente al daño inducido por la I/R en ratas (Lefer et al. 1997). Sin embargo, otros autores han demostrado para este radical un papel como mediador del daño secundario a la I/R (Mihm et al. 2001, Cuzzocrea et al. 2000).

Además, el ·NO se asocia a la muerte celular por apoptosis de forma dual. En concentraciones fisiológicas, la apoptosis se inhibe por nitrosilación de caspasa 3, efecto asociado a inhibición de la liberación del citocromo C por bloqueo del clavaje de Bcl-2 (caspasa 3-dependiente). En niveles altos, el ·NO actúa induciendo apoptosis, lo que podría estar mediado por peroxinitrito, el cual provocaría un cambio en la permeabilidad mitocondrial con la consecuente liberación de citocromo C, como también por daño al ADN y la consiguiente activación de PARS (poly (ADP-ribose) synthase) (Clemens 1999). Al mismo tiempo la pérdida de ATP conduce al metabolismo anaeróbico, con la consecuente acidosis y pérdida de la homeostasis celular. Subsecuentemente hay una pérdida de los gradientes iónicos a través de membrana, causando pérdida de Ca^{2+} y entrada de sodio. Esto a su vez origina edema seguido de la ruptura de enzimas y proteínas y finalmente muerte celular. La pérdida de enzimas y proteínas, especialmente aquellas de origen mitocondrial activa al sistema de complemento. Por otra parte la producción de radicales libres pude estimular la producción del factor activador de plaquetas (PAF) y leucotrieno B4, factores quimiotácticos que inducen la activación de neutrófilos, con la subsiguiente liberación de enzimas proteolíticos tales como mieloperoxidasa (MPO) y elastasa. La MPO cataliza la formación del altamente tóxico ácido hipocloroso (HOCl), un componente importante del daño oxidativo inducido por los neutrófilos. La elastasa puede degradar componentes importantes de matriz extracelular alterando la propiedad barrera de las células endoteliales, así como inducir la lisis celular. Cuando este proceso alcanza un cierto nivel, el daño puede ser irreversible.

Es ampliamente aceptado que el daño por isquemia reperfusión induce una respuesta inflamatoria. La isquemia produce lesión tisular fundamentalmente por anoxia, mientras que la reperfusión es esencialmente un fenómeno inflamatorio. La principal peculiaridad del fenómeno inflamatorio de la reperfusión está en los mediadores

bioquímicos que lo ponen en marcha, y en los que están implicados múltiples mediadores de la inflamación, plaquetas, leucocitos y el endotelio vascular, los cuales, al interaccionar, derivan en la lesión por reperfusión (Serracino-Inglott et al. 2001, Banga et al. 2005). Entre los mediadores inflamatorios descritos en la lesión por I/R destacan los radicales libres de oxígeno (Fan et al. 1999, Al-Mehdi et al. 1997) y las citoquinas (Lentsch et al. 2000), entre las cuales destacan las interleuquinas-1(IL-1), -6 (IL-6), -8 (IL-8), -10 (IL-10) y el factor de necrosis tumoral alpha (TNF-α) o las quimioquinas entre las cuales resaltan la proteína chimiotáctica de monocitos 1 (monocyte chemotactic protein 1, MCP-1) y la proteína inflamatoria de los macrófagos 2 (macrophage inflammatory protein 2, MIP-2).

Además, destacan por su papel en la respuesta inflamatoria, las moléculas de adhesión celular como la molécula de adhesión intracelular (Intercellular Adhesion Molecule, ICAM) y la proteína de adhesión vascular (VCAM) (Ranieri et al. 1999, von Hundelshausen et al. 2009). También se ha demostrado la participación de interleuquinas con propiedades antiinflamatorias que funcionan como reguladores del proceso inflamatorio que se desarrolla en la I/R, como IL-6, IL-13 y IL-10 (Ranieri et al. 1999). En el inicio de este proceso inflamatorio son determinantes los macrófagos alveolares (Jordan et al. 2000) y los mediadores inflamatorios que estos producen, entre los que destacan las citoquinas.

Las citoquinas proinflamatorias como IL-1, TNF-α o IF-γ así como el óxido nítrico y otros radicales libres pueden inducir apoptosis. Para ello emplean la unión y activación de receptores específicos así como la reducción en la expresión de la variante antiapoptótica del gen bcl-2 La unión de TNF a su receptor, TNFR, activa el dominio citosólico de muerte

celular asociado al receptor, (TRADD). Posteriormente se activan y reclutan tres proteínas más: RIP (proteína 1 de interacción con TNFR1), el dominio de muerte celular de Fas (FADD) y el dominio de muerte celular asociado al TNFR2 (TRAF2). Una vez reclutados los dominios de muerte, la cascada de acontecimientos es dual, ya que por un lado se puede inducir la apoptosis, o bien la respuesta inflamatoria, o ambas. En la primera opción, los dominios de muerte interaccionan con caspasas iniciadoras (principalmente caspasa 8) y en la vía inflamatoria es predominante la activación del NF-κB y 2 vías dependientes de quinasas: como p38 y JNK (quinasa del dominio n-terminal de c-Jun).

Diferentes estudios examinando los mecanismos celulares implicados en la inflamación han sugerido que la cascada de activación de quinasa podría jugar un papel importante en la respuesta inflamatoria. Las protein quinasas activadas por mitógenos (MAPKs) son una familia de serina/treonina protein quinasas que pueden actuar como mediadores en diferentes procesos biológicos fundamentales así como en las respuestas celulares a las señales externas de stress (Owens, Keyse 2007). Las tres cascadas de quinasas de stress mejor caracterizadas son la quinasa clásica activada por mitógenos (MAP) quinasa (ERK1/2), la protein quinasa activada por stress /c-Jun N-terminal protein quinasa y los enzimas de la familia p38. Estas cascadas de fosforilaciones transmiten y amplifican las señales extracelulares mediadas por receptor a través del citoplasma de la célula, activando la transcripción de factores nucleares los cuales inducen la expresión de diferentes genes diana (Owens, Keyse 2007).

Un importante papel de MAPK es su activación por una amplia variedad de estímulos incluyendo citoquinas, endotoxina y otros factores de stress, lo que conduce finalmente a la activación del factor de transcripción nuclear NF-κB. De forma similar al NF-κB, p38-MAPK ha sido implicada como un mediador crítico de la liberación de citoquinas proinflamatorias y regula positivamente la expresión de varios genes implicados

en la respuesta de fase aguda tales como TNF-α, IL-6 y otros enzimas inducibles implicados en la respuesta inflamatoria (Sun et al. 2011).

Estudios recientes sugieren que la apoptosis secundaria a la isquemia-reperfusión podría ser importante en el desarrollo de las alteraciones de la función pulmonar (Ng et al. 2005). Es aceptado generalmente que la apoptosis o 'muerte celular programada' juega un papel importante en el daño celular secundario a la reperfusión (Eefting et al. 2004) y se ha especulado que esto podría estar ligado a una disfunción en la liberación de citoquinas (El Azab et al. 2002).

Aunque los mecanismos de transducción que median la respuesta celular a estas condiciones de estrés no son totalmente conocidos, cada vez más parece haber un consenso generalizado atribuyendo un papel importante a las citoquinas como mediadores directos de la muerte por apoptosis. Las citoquinas proinflamatorias como IL-1, TNF-α o IF-γ así como óxido nítrico y otros radicales libres pueden inducir apoptosis. Para ello emplean la unión y activación de receptores específicos así como la reducción en la expresión de la variante antiapoptótica del gen Bcl-2. En respuesta a la isquemia, los tejidos producen factor de necrosis tumoral-α (TNF-α), que es un inductor de apoptosis (Rudiger, Clavien 2002). Se conocen varios receptores transmembrana para TNF (TNFR). De ellos, TNFR1 (p55) y 2 (p75) son los mejor conocidos, predominando TNFR1 en células de estirpe no hematopoyética. Coinciden en su estructura extracelular, si bien los dominios intracitoplásmicos son diferentes, lo cual sugiere además diferentes vías de señalización interna.

Una vez unido TNF a TNFR1, se activa el dominio de muerte celular asociado al receptor, situado dentro de su citoplasma (TRADD). Posteriormente se activan y reclutan tres proteínas más: RIP (proteína 1 de interacción con TNFR1), el dominio de muerte celular de Fas (FADD) y el dominio de muerte celular asociado al TNFR2 (TRAF2). En el caso de la unión TNF-TNFR2, se activa directamente TRAF2 que se asocia a TRAF1 (factor 1 asociado a TNF). Una vez reclutados los dominios de muerte, la cascada de acontecimientos es dual, ya que por un lado se puede inducir la apoptosis, o bien la respuesta inflamatoria, o ambas. En la primera opción, los dominios de muerte interaccionan con caspasas iniciadoras (principalmente caspasa 8) y en la vía inflamatoria es predominante la activación del NF-κB y 2 vías dependientes de quinasas: p38 y JNK (kinasa del dominio n-terminal de c-Jun).

Curiosamente, existe un mecanismo alternativo que potencia la acción del TNF y es la secreción aumentada por parte de los macrófagos tanto de TNFR1 como 2 en presencia de estímulos proinflamatorios (Mira et al. 1999). Sin embargo, este mecanismo es concentración-dependiente, pero saturable, de modo que concentraciones excesivas de receptores solubles pueden bloquear los efectos proinflamatorios y proapoptóticos del TNF.

La implicación de la apoptosis en el daño pulmonar agudo viene determinada por la observación de su relación con la activación inflamatoria (Matute-Bello et al. 2000), con el estrés oxidativo (Yulug et al. 2007), y con la con la ventilación mecánica (Vion et al. 2013). Así mismo se ha encontrado una activación de la apoptosis en relación al trauma vinculado a los procedimientos quirúrgicos (Oka et al. 1996, Delogu et al. 2000).

A pesar de que se habla de apoptosis como un modo de muerte celular que no se acompaña de inflamación, hay evidencia creciente de una relación estrecha entre ambos

fenómenos. La activación y amplificación de la respuesta inflamatoria vía TNF-α parece aumentar la apoptosis de neumocitos tipo II en algunos modelos de daño pulmonar agudo (Li et al. 2013). Las caspasas, juegan un papel central en este proceso de "suicidio ritual" (Thornberry, Lazebnik 1998).

Por otra parte en la apoptosis parecen jugar un papel regulador importante una serie de proteínas de la familia Bcl-2, entre las que se incluyen Bcl-2, que tiene un papel regulador antiapoptótico. Otras proteínas de esta familia son proapoptóticas y el resultado final va a depender del balance entre formas proapoptóticas y antiapoptóticas (Adams, Cory 1998).

El papel de la apoptosis en el daño pulmonar agudo se produce a distintos niveles, lo que determina en ocasiones efectos beneficiosos o perjudiciales. Por un lado la apoptosis de los neutrófilos, minimiza o limita la respuesta inflamatoria o inmunológica (Matute-Bello et al. 2000, Matute-Bello, Martin 2003). Por otro lado los fenómenos apoptóticos durante el ALI también afectan a las células epiteliales y endoteliales provocando un daño a la barrera alveolo-capilar (Bem et al. 2007), con consiguiente alteración en la permeabilidad a este nivel. Las citoquinas modulan el proceso de apoptosis, así pues en sitios donde existe inflamación pulmonar la apoptosis de los neutrófilos se encuentra atenuada (Matute-Bello et al. 1997) mientras que la de las células epiteliales está aumentada (Fujita et al. 1998).

El reclutamiento y la activación de neutrófilos debido al papel quimioatrayente de algunas citoquinas forman parte invariable del daño pulmonar agudo postoperatorio,

produciéndose una acumulación de estos en los pulmones, alcanzando los espacios aéreos. Posteriormente la apoptosis de los neutrófilos se produce para minimizar la liberación de oxidantes y enzimas que provocan lesión celular, así pues estos involucionan y son fagocitados por los macrófagos.

Se ha identificado un aumento en los niveles de GM-CSF (factor estimulante de colonias de granulocitos y monocitos), que tiene un papel fundamental al inhibir la apoptosis de neutrófilos, lo que prolonga y potencia el proceso inflamatorio (Matute-Bello et al. 2000). Por último se ha observado como la fagocitosis de estos neutrófilos apoptóticos afecta a la función de las células fagocíticas potenciando un cambio en la secreción de citoquinas proinflamatorias a anti-inflamatorias (Il-10), implicando por lo tanto a la apoptosis en la regulación de la respuesta inflamatoria e inmunológica (Matute-Bello et al. 2000).

La apoptosis de las células epiteliales alveolares ha sido implicada en varios modelos de ALI (Fujita et al. 1998, Hagimoto et al. 1997, Kawasaki et al. 2000, Kitamura et al. 2001, Vernooy et al. 2001). Normalmente las células epiteliales, restringen el movimiento de fluidos y proteínas desde el intersticio al alveolo, y transportan de forma activa Na y Cl desde los espacios alveolares, con el correspondiente movimiento de agua a través de canales especializados (acuaporinas). Esta lesión epitelial y endotelial por lo tanto va a alterar la permeabilidad, provocando un edema rico en proteínas.

Así mismo se ha observado un aumento de FAS-L soluble en el lavado bronco alveolar (LBA) de los pacientes que fallecieron por lesión pulmonar aguda (LPA)/SDRA. Además, se ha observado que este LBA era capaz de inducir apoptosis en células epiteliales, efecto que además desaparecía al utilizar inhibidores específicos de la activación de FAS/FAS-L (Matute-Bello et al. 1999). Por otro lado, en un modelo animal se ha observado

que la actividad del FAS-L, iniciando procesos de apoptosis caspasa-dependientes, está inversamente asociada con la integridad de la barrera epitelial y se ha relacionado con la incapacidad para reabsorber fluidos desde los espacios aéreos (Herrero et al. 2013). En este mismo aspecto, se ha observado que la instilación de FAS-L en pulmones de conejos puede causar hemorragia alveolar y aumentar la producción de citoquinas proinflamatorias como la IL-8, por parte de los macrófagos alveolares (Matute-Bello, Martin 2003). La activación dependiente de FAS-L no se produce de forma constante en toda la vía aérea, observándose una susceptibilidad diferenciada en los neumocitos de las unidades alveolares más distales (Nakamura et al. 2004). Por otro lado, los niveles de FAS-L en el lavado broncoalveolar se han asociado al daño multiorgánico y a un peor pronóstico en los pacientes con ALI y SDRA (Serrao et al. 2001, Albertine et al. 2002, Galani et al. 2010).

Durante la respuesta inflamatoria se produce la secreción de algunos mediadores solubles (FAS-L, TNF-α) que inducen aumento de los procesos apoptóticos (Serrao et al. 2001). Los receptores de la membrana celular comparten dominios (death domains) en ambas vías de señalización celular, lo que conlleva una interacción entre estas proteínas. Un ejemplo sería la estimulación de la vía del TNF-α, que determina una activación de fenómenos inflamatorios vía NF-κB así como un efecto proapoptótico por la vía extrínseca (Hsu et al. 1996). Así, la activación de ambos fenómenos comparte vías de regulación. Por otro lado, se ha observado un incremento de la actividad de BAX en los neumocitos alveolares de tipo 2 en los casos de ALI (Guinee et al. 1997), lo cual sugeriría una susceptibilidad aumentada de estas células epiteliales a la apoptosis. Parece que, en

definitiva, el balance de la actividad BAX/Bcl-2 determinaría la susceptibilidad de las células del epitelio alveolar a la apoptosis (Korsmeyer et al. 1993, Korsmeyer et al. 1995).

En resumen, la apoptosis en los procesos inflamatorios pulmonares, ha sido ampliamente investigada sugiriéndose su papel central en el daño pulmonar agudo, así como se ha sugerido que su inhibición tiene un papel protector en este proceso inflamatorio (Dosreis et al. 2004).

Recientemente, se ha sugerido que cambios en el perfil de expresión de microRNA podrían estar implicados en la respuesta inflamatoria y/o apoptótica a la I/R. Los microRNA (miRNAs) son pequeñas moléculas de ARN no codificante capaces de regular la expresión génica (Bartel 2004, Ambros, Chen 2007). Participan activamente en la modulación de importantes procesos celulares fisiológicos y están involucrados en la patogenia de enfermedades pulmonares, incluyendo enfermedades asociadas a una respuesta inflamatoria (Angulo et al. 2012).

Los miRNAs son transcritos por la RNA polimerasa II como largos primarios o pri-miRNA. Dentro del núcleo, ambos extremos del pri-miR son cortados por el complejo Drosha/DGCR8 dando lugar al precursor del miR maduro (pre-miR). Este es transportado activamente al citoplasma a través de Exportin-5, donde es procesado por el complejo Dicer/TRBP originando una molécula de ARN de doble cadena de 19 a 25 nucleótidos de largo. Una de las cadenas constituye el miR maduro, mientras que su complementario (denominado miR*) es generalmente degradado. El miRNA maduro es incorporado al complejo de silenciamiento inducido por RNA (RISC, RNA induced silencing complex) (Filipowicz et al. 2008). La actividad de los miRNAs se encuentra regulada de forma estricta, mediante el control de su transcripción, de las diferentes etapas de biogénesis y posteriormente de la función del miRISC (Krol et al. 2010).

La unión entre ARNm y miRNA se establece a través de cortas secuencias de reconocimiento de unos 6-7 nucleótidos de longitud, por lo que un mismo miRNA puede tener cientos de potenciales genes diana, y a su vez, un gen diana puede estar regulado por diferentes miRNAs, participando en un mecanismo complejo de regulación y comunicación intercelular (Pillai et al. 2007).

Teniendo en cuenta la enorme capacidad de los miRNAs para influir sobre la expresión de gran parte del genoma y el intrincado sistema de control en el que se encuentran inmersos, es comprensible que la desregulación de los mismos repercuta significativamente sobre la homeostasis del organismo. Un número creciente de miRNAs ha sido implicado en distintas enfermedades pulmonares, incluyendo patologías asociadas a procesos inflamatorios (Xie et al. 2012).

En este aspecto, se ha descrito que alteraciones en la expresión de determinados miRNAs podrían participar en la regulación del proceso inflamatorio y la reparación tisular en la lesión pulmonar aguda (LPA)/SDRA (Williams et al. 2007, Angulo et al. 2012). Por otro lado, diferentes investigadores han evidenciado la implicación de los miRNA en la respuesta inmune, innata y adquirida, indicando la existencia de una fuerte correlación entre la expresión de miRNA y las vías de señalización y receptores implicados en la respuesta inflamatoria, tales como NFκB (Oglesby et al. 2010, Jardim et al. 2009) y TLR (Oglesby et al. 2010).

Los miRNAs también pueden modular la expresión de sirtuinas que a su vez modulan (y son moduladas por) MAPKs y NFκB, que a su vez modulan la respuesta

inflamatoria, generándose así un círculo vicioso de generación y amplificación del daño celular y la consecuente pérdida de función.

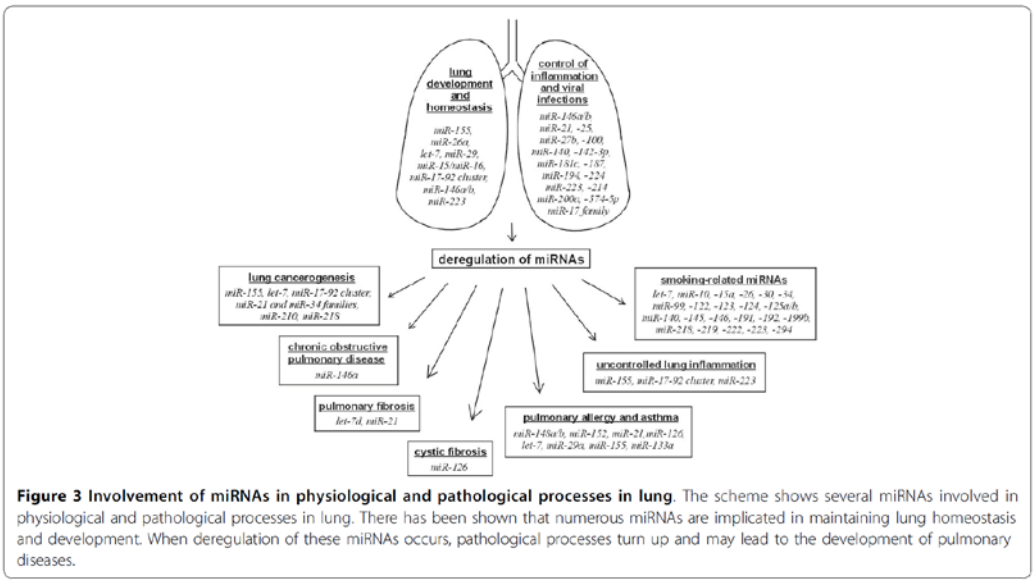

Figure 3 Involvement of miRNAs in physiological and pathological processes in lung. The scheme shows several miRNAs involved in physiological and pathological processes in lung. There has been shown that numerous miRNAs are implicated in maintaining lung homeostasis and development. When deregulation of these miRNAs occurs, pathological processes turn up and may lead to the development of pulmonary diseases.

Figura 1: miRNAs implicados en patologías pulmonares ("Involvement of microRNAs in physiological and pathological processes in the lung". Tomankova T. et al. Respiratory Research, 2010).

La ventilación unipulmonar

Durante la cirugía torácica, la ventilación unipulmonar (one lung ventilation, OLV) es una técnica ampliamente utilizada para facilitar el trabajo de los cirujanos durante las intervenciones torácicas que permite aislar un pulmón de la ventilación mecánica, siendo necesaria, entre otras situaciones, para aislar un pulmón enfermo del sano en abscesos y hemorragias, para controlar la distribución de la ventilación a un solo pulmón, o para el LBA unilateral; además, se utiliza para colapsar un pulmón en intervenciones quirúrgicas mediante video-toracoscopia o toracotomía, tanto para cirugía pulmonar como de otras estructuras torácicas como mediastino, esófago o aorta. Esto condiciona un colapso de ese pulmón que permite trabajar en ese hemitórax y la necesidad de mantener una adecuada ventilación del paciente con el pulmón contralateral.

En el tejido pulmonar, en respuesta a bajas concentraciones de oxígeno se produce una vasoconstricción arterial reactiva que desvía el flujo sanguíneo hacia las unidades alveolares con mayor presión de O_2 (vasoconstricción pulmonar hipóxica: VPH). Este es un mecanismo reactivo que depende directamente de la contracción refleja de la célula muscular lisa vascular en respuesta a la hipoxia, después de la despolarización ligada a canales de potasio y calcio (Post et al. 1995). Este reflejo adaptativo deriva la sangre arterial de las unidades peor ventiladas, a aquellas que tienen una ventilación más adecuada.

La ventilación unipulmonar provoca intensos cambios fisiopatológicos en la ventilación y la perfusión pulmonar ya que se produce un aumento del shunt fisiológico. Este, que en condiciones basales es de un 5% de la sangre debido a la circulación bronquial, llega a aumentar un 5-10% cuando se utiliza ventilación mecánica y hasta un 15-20% cuando se recurre a la OLV. Además, existen otros factores que también influyen en la relación ventilación/perfusión y que son propios de los diferentes procedimientos quirúrgicos que requieren ventilación unipulmonar, como la toracotomía y la anestesia. La instauración de la ventilación unipulmonar y el colapso del pulmón contralateral crea un shunt transpulmonar derecha-izquierda ya que todo el flujo que perfunde el pulmón no ventilado llega a las cavidades izquierdas sin oxigenar. Por tanto se produce un incremento de la diferencia alveolo arterial de O_2 y una disminución de la PaO_2. En esta situación se desencadenan unos mecanismos compensatorios activos, como la vasoconstricción pulmonar hipóxica (VPH), que reducen la proporción de flujo que llega al pulmón no ventilado y por tanto atenúan la caída de la PaO_2.

Se sabe que la VPH está modulada por numerosos factores, entre los que se incluyen aminas vasoactivas (catecolaminas, prostaglandinas, leucotrienos, serotonina, histamina, angiotensina, bradikinina, etc.), sin embargo, no se ha podido demostrar la liberación de ninguna de ellas en respuesta a la hipoxia como causante de la VPH. Por otro lado, la ventilación unipulmonar puede inducir una respuesta proinflamatoria, incluyendo liberación de citoquinas y migración de leucocitos en el pulmón ventilado (Zingg et al. 2010).

Durante la OLV se produce habitualmente, en mayor o menor grado, un daño pulmonar agudo (Acute Lung Injury; ALI) que eleva la morbimortalidad postoperatoria en cirugía de resección pulmonar (Gothard 2006). Por lo tanto, la OLV va a condicionar distintos estímulos implicados en el daño pulmonar agudo (ALI) como barotrauma, volutrauma, atelectrauma o hiperoxia que actúan sobre el pulmón ventilado o dependiente.

En el pulmón sometido a cirugía, no dependiente, tiene lugar una hipoperfusión relativa como consecuencia de la VPH y, posteriormente, un síndrome de isquemia-reperfusión (I/R) en el tejido pulmonar remanente, tras la re-expansión de ese tejido a la finalización del procedimiento. Estos estímulos dañan la membrana alveolo capilar, favoreciendo el edema alveolar y la microtrombosis en los capilares alveolares (Slinger 2003, Tremblay et al. 2002) y desencadenan una respuesta inflamatoria aguda con liberación de interleuquina-8 (IL-8), interleuquina-1 (IL-1) o factor de necrosis tumoral (TNF-α) (Baudouin 2003, Funakoshi et al. 2004) .

La participación de los procesos inflamatorios durante la OLV y el papel de las citoquinas también parecen fundamentales. Así pues, se ha observado que durante las cirugías con OLV existe un proceso inflamatorio, que afecta fundamentalmente al pulmón dependiente, con reclutamiento de neutrófilos y secreción de moléculas proinflamatorias

como IL-8, IL-1α, TNF-α, especies reactivas de oxígeno, eicosanoides o complemento. En general, se han observado respuestas inflamatorias de carácter agudo en todos los contextos quirúrgicos que implican OLV, cuantitativamente superiores a las observadas en otras cirugías que conllevan ventilación bipulmonar (Sakamoto et al. 1994, Grichnik, D'Amico 2004). Así, se podría hipotetizar que el daño pulmonar en respuesta a la CRP podría tener dos componentes, uno secundario a la propia cirugía y los procesos de I/R que conlleva. También, se podría hipotetizar que además del daño pulmonar que se produce por la respuesta inflamatoria sistémica asociada a la cirugía, el daño pulmonar durante la CRP podría tener dos componentes: uno secundario a la ventilación mecánica de un pulmón en condiciones desfavorables (hiperoxia, altas presiones vía aérea, baja complianza pulmonar) y otro a los fenómenos de I/R que se producen en el pulmón que durante la OLV no es ventilado y sufre una disminución del aporte de oxígeno.

Efectos sistémicos de la lesión por isquemia/reperfusión

Se ha demostrado que el daño por I/R no solo afecta a órganos locales, sino que causa una respuesta inflamatoria que puede afectar también a órganos distantes. El hígado es un órgano fundamental, que juega un papel central en el mantenimiento de la homeostasis del organismo. Se ha descrito que el hígado es un órgano particularmente sensible al incremento de los niveles circulantes de mediadores inflamatorios secundario a la I/R de órganos lejanos tales como riñón (Kielar et al. 2002, Kelly 2003), intestino (Horie, Ishii 2001, Golab et al. 2009) o músculo esquelético (Kanoria et al. 2006, Lai et al. 2006). Por otro lado en estudios experimentales realizados en conejos se ha observado que la I/R

pulmonar induce un daño hepático caracterizado por la infiltración de neutrófilos activados y un aumento de los niveles de radicales libres de oxígeno (Esme et al. 2006).

En estudios anteriores de nuestro grupo hemos observado que la I/R pulmonar modificaba los niveles de los mediadores inflamatorios estudiados tanto en tejido pulmonar y lavado broncoalveolar (LBA) como en plasma (Casanova et al. 2011, Simon Adiego et al. 2011, Simon et al. 2012), sugiriendo que aunque no es frecuente observar alteraciones clínicas hepáticas secundarias a la cirugía torácica, sin embargo es posible que la I/R pulmonar pueda inducir alteraciones subclínicas del hígado secundarias a la I/R pulmonar, complicando la evolución clínica de los pacientes.

Modulación de la lesión por isquemia/reperfusión

De cara a proteger los órganos frente a los fenómenos de isquemia-reperfusión, se han intentado establecer distintos tipo terapias preventivas para atenuar este daño celular, entre ellas se encuentran las técnicas de precondicionamiento. El precondicionamiento puede realizarse de forma mecánica provocando pequeños ciclos de isquemia, denominado precondicionamiento isquémico, con buenos resultados tanto a nivel pulmonar (Soncul et al. 1999) como hepático (Hardy et al. 1996). Otro modo sería el precondicionamiento farmacológico.

Se ha demostrado la eficacia a nivel de diferentes órganos, como en corazón a través de la utilización de antagonistas del calcio como el amlodipino (Ahmed et al. 2009) o con ketoralaco (Pichardo et al. 1994), o también en caso del riñón, donde se ha observado en cerdos una mejora en la función renal post-isquemia cuando se utilizó nimodipino (Froba et al. 2008). El sevoflurano, al igual que otros halogenados (precondicionamiento anestésico), también tiene un papel importante debido a sus efectos citoprotectores.

En 1986, Murry y cols. describieron el fenómeno llamado precondicionamiento isquémico (PCI), que proporciona uno de los métodos más potentes para atenuar la lesión por isquemia-reperfusión (Murry et al. 1986). Este trabajo de isquemia-reperfusión en corazón de perros se confirmó después en otras especies animales y en otros órganos (Pasupathy, Homer-Vanniasinkam 2005).

El PCI es efectivo al cabo de unos minutos, lo que sugiere que los mediadores responsables de este efecto existían previamente. Algunos agonistas de liberación local como la adenosina, bradikinina, catecolaminas y opioides disparan esta reacción de protección a través de varios receptores de la membrana celular relacionados con la proteína G.

El efector último del precondicionamiento no es totalmente conocido, pero parece estar muy relacionado con los canales mitocondriales de potasio dependientes del ATP (Garlid et al. 2003). En 1993 se describió una segunda ventana de protección (SWOP) desarrollada 12 a 24 horas tras el estímulo inicial de precondicionamiento (Kuzuya et al. 1993). A diferencia del PCI temprano, que es de corta duración (2-3 horas), el SWOP protege activamente durante 48-96 horas, aunque con una eficacia menor y a través de diferentes mecanismos.

También se ha demostrado que los episodios de PCI repetidos no aumentan el grado ni la eficacia de la protección (Pasupathy, Homer-Vanniasinkam 2005). En conjunto el fenómeno de PCI es ubicuo. Las dos formas de PCI emplean mecanismos endógenos en varios órganos y sistemas protegiendo de una manera notable a los tejidos frente a la lesión

de isquemia-reperfusión. Algunos agonistas fisiológicos como la adenosina, bradikinina, catecolaminas y opioides disparan la respuesta de PCI precoz.

El PCI tardío se inicia con adaptaciones genéticas para producir sustancias nuevas o por lo menos en cantidades superiores a lo normal, desencadenando una situación fisiológica anormal. Además hay vías neurógenas que también transmiten importantes señales protectoras.

Con todo ello los tejidos precondicionados quedan mejor equipados para soportar la lesión de isquemia-reperfusión (Pasupathy, Homer-Vanniasinkam 2005). Durante la isquemia los tejidos pre-tratados muestran una reducción de las necesidades de energía, una mayor conservación de los sustratos energéticos, disminución del metabolismo y una mayor eficacia en la regulación del balance iónico y ácido-base. Tras la reperfusión los tejidos preparados con IP muestran una disminución del estrés oxidativo, de la activación de neutrófilos, de la producción de citoquinas y de la apoptosis y mejorar la microcirculación.

Por otro lado, como ya se ha indicado, en los últimos años se ha sugerido que el precondicionamiento anestésico (PCA) podría ejercer efectos citoprotectores frente a los fenómenos inflamatorios y apoptóticos secundarios a isquemia-reperfusión en diversos órganos como el corazón, riñón, hígado y cerebro. Los mecanismos precisos por los que estos agentes ejercen la protección frente a la isquemia, no parecen deberse en exclusiva al mejor balance entre el aporte y el consumo de oxígeno derivado de sus efectos hemodinámicos.

A nivel miocárdico, se ha objetivado además que los gases halogenados producen la apertura de canales de K^+ dependientes de ATP mitocondriales y sarcolémicos (Tanaka et al. 2004a), al tiempo que reducen la sensibilidad de éstos para cerrarse por ausencia de

ATP (Han et al. 1996), aumentando así la probabilidad de que permanezcan abiertos. Esta apertura de los canales de K dependientes de ATP va a provocar en el músculo liso de los vasos coronarios una vasodilatación, lo cual va a mejorar el aporte de oxígeno, y en cierto modo protege durante la isquemia cuando la producción de ATP se ve reducida. Además el sevoflurano induce un incremento de la circulación colateral mediado por los canales de K calcio dependientes (Novalija et al. 1999). Se ha demostrado con anterioridad que los anestésicos halogenados podían proteger contra el daño postreperfusión en el corazón (Redel et al. 2009), cerebro (Lin et al. 2009) e hígado (Imai et al. 1996).

A nivel pulmonar existen menos investigaciones, aunque se ha comprobado en el pulmón del conejo que administrar isofluorano o sevoflurano antes de la isquemia atenúa el aumento de la permeabilidad vascular y la relación peso húmedo/seco, disminuye los niveles de TNF-α así como los metabolitos del pulmón reperfundido después de la isquemia (Liu et al. 2000).

El sevoflurano también ha demostrado, en un modelo in vitro, disminuir el aumento de la permeabilidad endotelial pulmonar inducido por TNF-α, asociado a un efecto inhibidor sobre la vía de las MAPK (Mapk-p38) (Sun et al. 2011). Los efectos protectores del sevoflurano a nivel pulmonar en este caso, parecen ponerse en relación con una atenuación de la respuesta inflamatoria y la lesión asociada.

El efecto protector pulmonar del PCA con sevoflurano, también se ha observado al atenuar la lesión pulmonar que puede desencadenarse tras la I/R de los miembros inferiores en cirugía aórtica (Kalb et al. 2008). Así mismo, en un estudio en humanos sometidos a

bypass cardiopulmonar, la administración de sevoflurano ha demostrado disminuir los niveles de neutrófilos y citoquinas proinflamatorias a nivel pulmonar (Cho et al. 2009).

Recientemente nuestro grupo ha demostrado el efecto beneficioso del sevoflurano en un modelo de lesión pulmonar por isquemia-reperfusión en un modelo de autotrasplante pulmonar en cerdos (Casanova et al. 2011), sugiriendo la potencial utilidad que tendría el sevoflurano en el manejo anestésico de los pacientes sometidos a cirugía pulmonar. De acuerdo con esto, estudios comparativos entre sevoflurano y propofol en cirugía torácica, han asociado la administración de sevoflurano con un menor aumento de mediadores inflamatorios así como a un menor índice de complicaciones postoperatorias (De Conno et al. 2009, Sugasawa et al. 2012), sugiriendo un posible papel inmunomodulador del sevoflurano.

Los anestésicos locales (AL) son usados tradicionalmente como anti-arrítmicos y anestésicos por su capacidad para bloquear los canales de Na, sin embargo, poseen efectos importantes en otros contextos clínicos (neuroprotector, glaucoma de ángulo cerrado, íleo postquirúrgico), a dosis mucho menores que las utilizadas para bloquear los canales de Na. También producen un efecto antiinflamatorio, especialmente sobre la inmunidad celular (sobre polimorfonucleares, monocitos y macrófagos). Esta disminución de la respuesta inflamatoria por los AL ocurre a través de distintos mecanismos entre los que se encuentran la modulación de la producción, liberación y actividad de las citoquinas, la disminución de la adhesión y quimiotaxis de los neutrófilos, así como reduciendo la producción de superóxido (Taniguchi et al. 2000). Estos efectos antiinflamatorios también se han observado en modelos experimentales de isquemia reperfusión miocárdica y en cirugía colorrectal en humanos. En cuanto al pulmón, estudios experimentales demuestran que la administración de ropivacaína o lidocaína atenúa el daño pulmonar inducido por

endotoxina o HCl pero no se ha estudiado el impacto de los AL sobre el IRI que se observa en la CRP.

Es aceptado que las alteraciones en el glicocálix podrían jugar un papel importante en el daño secundario a la I/R (Mulivor, Lipowsky 2004, Annecke et al. 2010). En situaciones fisiológicas, el endotelio vascular está recubierto por el glicocálix. Este consiste en una capa de glicosaminoglicanos, altamente sulfatados unida a diferentes proteoglicanos; aproximadamente, entre el 50-90% son heparan sulfatos.

El glicocálix también se compone de una amplia gama de enzimas y proteínas que regulan la adhesión de leucocitos y trombocitos. Así, el glicocálix sirve como una barrera de permeabilidad vascular mediante la inhibición de la coagulación y la adhesión de los leucocitos. Juega un papel importante en la regulación de la resistencia vascular, permeabilidad y el reclutamiento de leucocitos (Weinbaum et al. 2007, Mulivor, Lipowsky 2002). La interrupción de esta estructura, por tanto, podría tener efectos perjudiciales. Investigaciones recientes, tanto clínicas como experimentales, han demostrado que el glicocálix es severamente alterado tras I/R (Mulivor, Lipowsky 2004, Rubio-Gayosso et al. 2006). Se ha sugerido que estos efectos podrían estar modulados por un sistema complejo de señales intracelulares sensible a modificaciones en los niveles de mediadores inflamatorios, incluyendo citoquinas, y/o de estrés oxidativo.

Se ha descrito que en corazón, niveles mínimos de oxígeno eran suficiente para causar la degradación del glicocálix; la degradación del glicocálix también puede ser activada por estímulos inflamatorios, tales como el factor de necrosis tumoral. Los efectos

deletéreos de la I/R sobre el glicocálix pueden ser prevenidos, al menos en parte, por tratamiento con hidrocortisona (Chappell et al. 2007), antitrombina (Chappell et al. 2009), precondicionamiento isquémico (Beresewicz et al. 1998) o los anestésicos halogenados (Annecke et al. 2010). Sin embargo, si la estructura del glicocálix está alterada en la resección pulmonar y su posible modulación por PCA, no ha sido investigada.

La respuesta inflamatoria, el estrés oxidativo y la regulación de factores de transcripción, han sido asociados al daño secundario a la I/R. Recientemente, se ha sugerido que alteraciones en el glicocálix y en la expresión de miRNA también podrían estar implicados en la respuesta a la I/R. El conocimiento de la fisiopatología del daño secundario a la cirugía pulmonar, permitiría el desarrollo de terapias biológicas encaminadas al bloqueo de los fenómenos en él observados. En este aspecto, se han intentado establecer distintos tipo terapias preventivas para atenuar este daño celular (Técnicas de precondicionamiento).

Las técnicas de precondicionamiento podrían convertirse en una poderosa herramienta para el manejo de pacientes sometidos a intervención quirúrgica en la que se prevea una necesaria inducción de una isquemia y consecuente reperfusión. Sin embargo, son necesarias más investigaciones para determinar tanto los parámetros, como las condiciones más útiles para su aplicación clínica generalizada.

HIPÓTESIS Y OBJETIVOS

"Si quieres hacer un paso hacia adelante, debes perder el equilibrio durante un momento".

- *M. Gramellini*

El tiempo de isquemia caliente en determinados procedimientos quirúrgicos en los que el flujo sanguíneo al parénquima pulmonar queda atenuado por la vasoconstricción pulmonar durante un tiempo superior a una hora, afectará a la recuperación del adecuado funcionamiento del órgano, así como de forma sistémica, debido a la liberación de mediadores de la inflamación al torrente sanguíneo, a la evolución postoperatoria.

La ventilación unipulmonar realizada para determinados procedimientos quirúrgicos afectará no solo el funcionamiento del órgano, sino que también producirá una respuesta inflamatoria sistémica debido a la liberación de mediadores proinflamatorios al torrente sanguíneo. Sería posible modular la respuesta inflamatoria pulmonar aguda y con ello la respuesta sistémica mediante técnicas de precondicionamiento: PCI y/o PCA.

La apoptosis secundaria a la isquemia-reperfusión podría ser importante en el desarrollo de las alteraciones de la función pulmonar que podría estar ligado a una disfunción en la liberación de citoquinas y/o radicales libres. Esta respuesta podría ser modificada mediante técnicas de precondicionamiento.

El objetivo general de este trabajo, fué investigar el posible papel de los mediadores proinflamatorios y apoptóticos en el daño secundario a la isquemia-reperfusión pulmonar y determinar si estos cambios van acompañados por modificaciones en alteraciones del glicocálix y/o la expresión de miRNAs. Además se investigó la posible modulación de este daño mediante técnicas de precondicionamiento o la administración de lidocaína

Para ello nos planteamos:

1. Determinar la secuencia de liberación de mediadores proinflamatorios tanto a nivel local como sistémico, tras diferentes períodos de tiempo de isquemia caliente, y post reperfusión.
2. Determinar si estos cambios van acompañados por modificaciones en el grado de apoptosis.
3. Determinar si estos cambios van acompañados por modificaciones en la expresión de miRNAs y/o alteraciones del glicocálix.
4. Determinar, el posible efecto beneficioso del precondicionamiento sobre los parámetros indicados anteriormente.

MATERIALES Y MÉTODOS

"El estudio y, en general, la búsqueda de la verdad y la belleza conforman un área donde podemos seguir siendo niños toda la vida".

- *A. Einstein*

Los experimentos incluidos en esta tesis se han realizado de la siguiente forma:

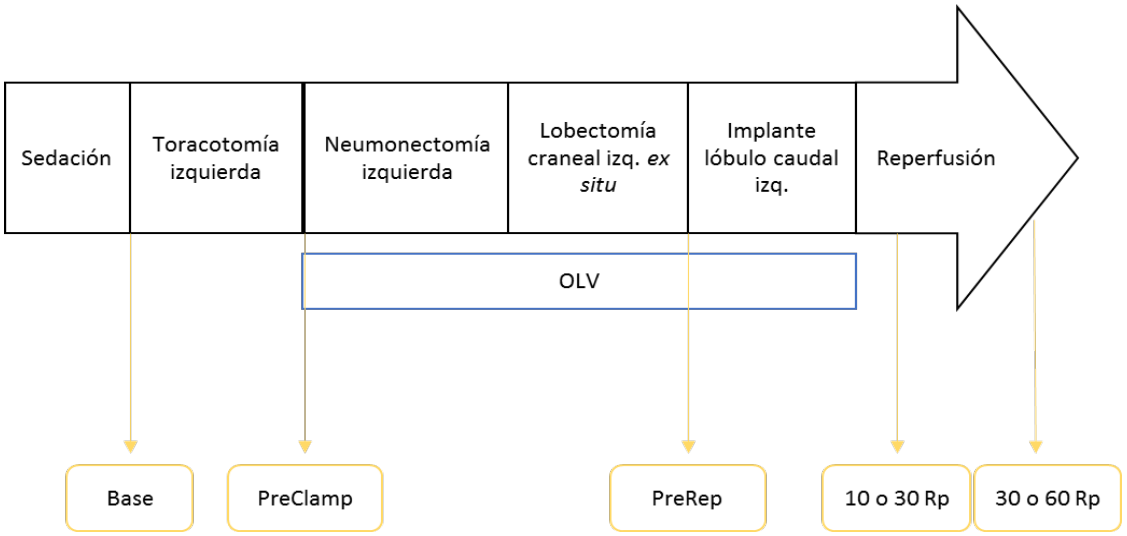

Figura 2: Representación esquemática del modelo experimental de auto-trasplante (I, II, IV, V y VI)

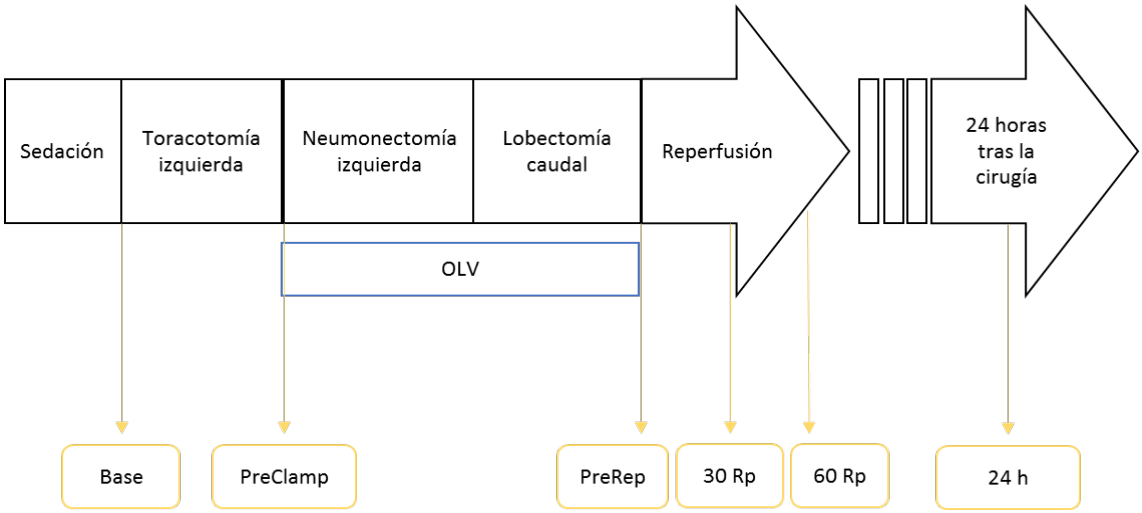

Figura 3: Representación esquemática del modelo experimental de ventilación unipulmonar (III)

Determinaciones bioquímicas:

- **EDEMA** (wet/dry ratio)
- **Expresion de mRNA (RT- PCR)**: IL-1β, IL-10, TNF-α, MCP-1, eNOS, iNOS, NFκB.
- **Expresión de miRNA (RT**-PCR): let-7d, 16,21,103, 107, 126, 127, 142-5p, 145, 146ª, 152, 155, 182, 192, 223
- **Expresión de proteínas (Western Blott)**: IL-1β, IL-10, TNF-α, eNOS, iNOS, BAD, BAX, Bcl-2, caspasa 3, VCAM
- **Niveles plasma y/o tejido (ELISA)**: IL-1β, IL-2, IL-10, TNF-α, MMP-2, MMP-3, MMP-9, Proteína C reactiva, Ferritina, Caspasa 3, Caspasa 9, Syndecan-1, Heparan sulfato, MPO, ICAM
- **Niveles de NO** (Reacción de Griess)

RESULTADOS

"La ciencia tiene las raíces amargas pero muy dulces los frutos."

- *Aristóteles*

Exponemos a continuación los resultados más significativos:

1. La cirugía de resección pulmonar indujo un aumento significativo de los niveles de citoquinas proinflamatorias en el pulmón. Por el contrario disminuyó los niveles de citoquinas antiinflamatorias. Estos efectos fueron también observados a nivel sistémico. (Artículos I, II, III, IV, V, VI y VII).
2. La cirugía de resección pulmonar indujo un aumento significativo de los niveles de mediadores proapoptóticos (Artículos I, II, III, IV, V, y VII).
3. La OLV *per se* incrementó significativamente los niveles de mediadores proinflamatorios y apoptóticos (Artículo III)
4. La cirugía de resección pulmonar modificó el perfil de expresión de miRNAs (Artículo IV). Estas alteraciones fueron aún más evidentes tras la reperfusión.
5. Se observaron alteraciones en la integridad del glicocálix tanto antes como después de la reperfusión (Artículos IV y V)
6. Tanto el precondicionamiento (PCI o PCA), como la administración de lidocaína redujeron significativamente las alteraciones anteriormente mencionadas (Artículos II, III, IV, V, VI y VII).

DISCUSIÓN

"Cambia tus pensamientos, y cambiará tu mundo."

- *Norman Vincent Peale*

La interrupción de la circulación pulmonar durante un tiempo prolongado (isquemia) y el posterior restablecimiento de la misma (reperfusión) activa procesos moleculares que causan daño pulmonar y desencadenan una respuesta inflamatoria sistémica con trascendencia en la práctica clínica (King et al. 2000, Padilla, Padilla 2004). En cirugía torácica, el proceso terapéutico que clásicamente se relaciona con la I/R es el trasplante pulmonar, que conlleva isquemias muy prolongadas del órgano. En estas condiciones, el órgano se mantiene en condiciones de hipotermia, sin embargo, cada vez con mayor frecuencia, en los últimos años, se han desarrollado y extendido procedimientos técnicos en los que la isquemia pulmonar no se realiza en hipotermia, sino en normotermia (isquemia caliente), en isquemia tibia (manteniendo el órgano a 12-14º C de temperatura), o en una combinación de ambas, durante un periodo más o menos prolongado de tiempo. Este es el caso de las cirugías en las que se realiza reconstrucción de la arteria pulmonar (angioplastia) y del trasplante lobar de donante vivo. En estos modelos el daño debido a I/R es mayor del que se observa cuando se pueden aplicar los protocolos que permiten enfriar el órgano durante el periodo isquémico. Por ello hemos desarrollado diferentes modelos experimentales que se asemejan mucho a estas situaciones clínicas: el pulmón es sometido a un proceso de isquemia caliente prolongada, durante un procedimiento de autotrasplante o de resección pulmonar, en un mamífero de gran tamaño (cerdo) de comportamiento biológico muy parecido al del ser humano.

Como se ha comentado en la introducción, una de las principales alteraciones debidas a la I/R consiste en un aumento de la respuesta inflamatoria tanto a nivel local como a nivel sistémico. De acuerdo con ello, en nuestros trabajos hemos observado una

alteración de la respuesta inflamatoria, tanto a nivel local como a nivel sistémico, en todos los modelos quirúrgicos analizados.

A nivel local hemos podido observar que la isquemia *per se* es capaz de inducir un incremento en los niveles de citoquinas pro-inflamatorias como TNF-α o IL-1β y un descenso en los niveles de IL-10, una citoquina anti-inflamatoria. Este incremento de los mediadores pro-inflamatorios, acompañado por el descenso de los anti-inflamatorios, indica un desbalance de la respuesta inflamatoria y refleja un posible daño tisular. Todas las alteraciones descritas se observaron tanto en los modelos de resección pulmonar *ex situ* como en las resecciones lobares *in situ.* Además, y de acuerdo con trabajos previos tanto de nuestro grupo (Simon Adiego et al. 2011, Casanova et al. 2011), como de otros grupos (de Perrot et al. 2003) hemos observado que, tras la reperfusión, se evidenciaba un ulterior incremento de estas alteraciones, apoyando la hipótesis de que el daño originado a los tejidos ocurre no solo durante la fase hipóxica o isquémica, sino también durante la reoxigenación del tejido (Wilkins et al. 1994, Shinozawa, Koike 2003).

Los cambios en los niveles de mediadores inflamatorios en pulmón fueron acompañados de modificaciones en los niveles plasmáticos de los mediadores inflamatorios analizados, sugiriendo que la I/R pulmonar además del daño local, puede inducir un daño sistémico. De acuerdo con esto, en este estudio hemos observado que la I/R pulmonar indujo una respuesta inflamatoria hepática. Estos resultados sugieren que la I/R pulmonar no es solo un proceso local, sino que como consecuencia de la entrada de mediadores inflamatorios en la circulación sistémica el hígado se ve afectado. Estos resultados confirman resultados previos de otros autores describiendo la afectación de un órgano lejano debido a procesos de I/R (Horie, Ishii 2001, Golab et al. 2009).

Pocos estudios han investigado el efecto de la I/R pulmonar sobre órganos lejanos. Esme et al., han descrito un incremento de marcadores de estrés oxidativo y de MPO en hígado de conejo, en respuesta a I/R pulmonar (Esme et al. 2006). De acuerdo con esto, en nuestro grupo hemos observado un aumento de los niveles de citoquinas proinflamatorias en el hígado de cerdos sometidos a cirugía de resección pulmonar. Además hemos observado una disminución de la expresión de citoquinas antiinflamatorias, sugiriendo que la I/R pulmonar puede inducir una alteración en el equilibrio entre mediadores proinflamatorios/antiinflamatorios, a favor de los primeros. Un efecto similar fue observado cuando se determinaron los niveles de marcadores de apoptosis, observándose un incremento de marcadores proapoptóticos frente a los antiapoptóticos. Se podría especular que la I/R pulmonar desencadenaría una cascada proinflamatoria y/o apoptótica que se traduciría en un daño a órganos remotos como el hígado.

En resumen, nuestros resultados parecen indicar que la I/R pulmonar no es solo un fenómeno local sino que a través de la liberación de mediadores proinflamatorios a la circulación sistémica puede inducir una respuesta sistémica que afectaría a otros órganos como el hígado. Esto sugiere, que el tratamiento utilizado se debería dirigir a ambas respuestas, local y sistémica.

Diferentes estrategias han sido descritas para reducir el daño pulmonar en cirugía torácica. La utilización de estas herramientas va dirigida a disminuir la elevada morbimortalidad asociada a este tipo de intervenciones que se relacionan fundamentalmente con una exagerada respuesta inflamatoria pulmonar y sistémica. Las técnicas de precondicionamiento, el uso de ventilación protectora, la asociación de técnicas

regionales para la analgesia, evitar concentraciones elevadas de oxígeno y un adecuado uso perioperatorio de fluidos han sido claves para mejorar el pronóstico de los pacientes sometidos a este tipo de cirugías. De cara a proteger los órganos frente a los fenómenos de I/R, se han intentado establecer distintos tipo terapias preventivas para atenuar este daño celular. Como ya se ha indicado, en los últimos años se ha sugerido que el PCI y/o PCA podría ejercer efectos citoprotectores frente a los fenómenos inflamatorios y apoptóticos secundarios a I/R en diversos órganos como el corazón, riñón, hígado y cerebro (Imai et al. 1996, Pasupathy, Homer-Vanniasinkam 2005, Redel et al. 2009, Lin et al. 2009). En particular, se ha observado que el PCI actúa activando tanto las vías neurogénicas como las vías humorales (Tapuria et al. 2008) sugiriéndose una modulación por su parte de las vías de señalización activadas por citoquinas como el TNF-α (Ates et al. 2002, Waldow et al. 2005), la IL-1 (Waldow et al. 2005) o el MCP-1 (Wei et al. 2011). También se ha observado que el PCI modula la expresión de NF-κB e iNOS (Li et al. 2004).

Por otro lado, estudios anteriores han demostrado que los anestésicos halogenados pueden modular la respuesta inflamatoria disminuyendo la migración (Tait et al. 1993) y la adhesión (Kowalski et al. 1997) de los neutrófilos. Asimismo, se ha comprobado que estos anestésicos poseen la capacidad de inhibir la liberación de TNF-α en un cultivo de células mononucleares (Mitsuhata et al. 1995, Tassiopoulos et al. 1998). Sin embargo, el posible efecto protector del precondicionamiento en la cirugía de resección pulmonar no está totalmente establecido.

Recientemente nuestro grupo ha demostrado un efecto beneficioso del precondicionamiento, tanto el isquémico (Simon et al. 2012) como el anestésico con sevoflurano (Casanova et al. 2011), frente a la respuesta inflamatoria secundaria a la I/R pulmonar. Sin embargo, estos estudios anteriores focalizaban su atención sobre los efectos locales del precondicionamiento. Por ello, en este trabajo hemos dirigido nuestra atención

al efecto que estos dos tipos de precondicionamiento tenían sobre las alteraciones sistémicas y hepáticas debidas al IRI.

Hemos observado que el PCI pulmonar redujo ambas respuestas, inflamatoria y apoptótica, inducidas en el hígado por la IR pulmonar, sugiriendo que el PCI del pulmón podría ejercer un efecto protector frente al daño secundario a la I/R, tanto a nivel local como sistémico.

El PCA con sevoflurano también disminuyó la respuesta inflamatoria sistémica secundaria a la I/R pulmonar. Sin embargo, el mecanismo por el que el sevoflurano ejerce su efecto protector no está totalmente aclarado. Ha sido descrito que los anestésicos volátiles pueden modular la respuesta inflamatoria (Shayevitz et al. 1991) y proteger frente al daño oxidativo (Johnson et al. 1996). De acuerdo con esto, hemos observado que el sevoflurano, de forma semejante a lo observado en pulmón (Casanova et al. 2011), redujo la expresión de las citoquinas proinflamatorias, TNF-α e IL-1β, en hígado. Además, el PCA también redujo la expresión de MCP-1 y NFκB y disminuyó la expresión de la citoquina antiinflamatoria, IL-10. Esto sugiere que el PCA con sevoflurano contribuye al mantenimiento del balance entre citoquinas pro y anti- inflamatorias, también en el hígado

Nuestros resultados están por lo tanto de acuerdo con los estudios anteriores y confirman la capacidad, tanto del PCI como del PCA, de modular la respuesta inflamatoria sistémica.

Como ya se ha indicado anteriormente, la I/R puede activar los procesos de muerte celular por apoptosis. La hipoxia, el aumento de los niveles de Ca, el NO y/o las citoquinas

parecen estar implicados en este proceso y son considerados como señales apoptóticos. Las caspasas juegan un papel central en la transducción de las señales apoptóticas. Entre las citoquinas, el TNF-α es considerado como uno de los mediadores extrínsecos de apoptosis más importantes. Por otro lado, el NFκB parece jugar un papel central modulando la apoptosis secundaria a la I/R, a través de un mecanismo que implica la inhibición, hipoxia-dependiente, de los sensores de oxígeno (Cummins et al. 2006). De acuerdo con esto, hemos observado que el aumento de la expresión hepática de TNF-α y NFκB en respuesta a la I/R pulmonar, iba acompañado de un incremento muy significativo de la actividad de caspasa 3 en hígado, sugiriendo que el aumento de la apoptosis en hígado podría ser consecuencia del daño inflamatorio. La reperfusión con sevoflurano redujo significativamente la actividad de caspasa 3, sugiriendo que el PCA con sevoflurano no solo protege frente al desbalance inflamatorio sino también frente a la apoptosis hepática secundaria a la I/R en un órgano lejano como el pulmón. El mecanismo por el que el sevoflurano ejerce este efecto protector frente a la apoptosis en hígado secundaria a la I/R pulmonar no está totalmente aclarado e investigaciones posteriores son necesarias.

Este trabajo experimental se ha plasmado en la realización de un ensayo clínico del que ya tenemos resultados previos (artículo VII).

Por otro lado, y de acuerdo con trabajos previos de otros autores, sugiriendo un efecto antiinflamatorio para los anestésicos locales (Taniguchi et al. 2000), en este trabajo hemos observado que la administración de lidocaína fue capaz de reducir la respuesta inflamatoria secundaria al daño por I/R pulmonar. Este efecto beneficioso fue aparente tanto en el daño debido a OLV y resección pulmonar como en el daño debido a trasplante lobar.

No está claro el mecanismo de acción por el cual la lidocaína puede ejercer su efecto beneficioso. Hollmann et al. demostraron que la lidocaína es capaz de inhibir de manera reversible los receptores acoplados a las proteínas G (Hollmann et al. 2001). Una inhibición de estos receptores por parte de la lidocaína podría tener consecuencias positivas en la respuesta inflamatoria y explicar, al menos en parte, los efectos de la administración de lidocaína observados en nuestros trabajos.

Lahat y cols. demostraron en cultivos celulares de células T que la lidocaína, a concentraciones clínicamente relevantes, reduce de forma dosis dependiente los niveles de mRNA de TNF-α lo cual indica que la disminución de la secreción de TNF-α ocurre a nivel transcripcional inhibiendo la expresión de mRNA, por un mecanismo no del todo determinado pero que se debe al menos en parte a la inhibición de la translocación nuclear de NF-κβ (Hagimoto et al. 1997). El paso de NF-κβ al núcleo regula la activación de la transcripción de gran cantidad de genes proinflamatorios incluyendo TNF-α

Papavlassopoulos y cols. han demostrado que canales específicos de potasio (maxiK) inician la vía de señalización de NF-κβ y que el bloqueo farmacológico de estos canales impide la degradación de Iκβ y la posterior translocación nuclear de NF-κβ, subrayando así el papel central que tienen estos canales en esta vía de señalización (Papavlassopoulos et al. 2006). Mientras que Kindler y cols. han demostrado que los anestésicos locales de tipo amida pueden bloquear estos canales de potasio (Kindler, Yost 2005), lo cual puede formar parte de uno de los mecanismo intracelulares de inhibición de la lidocaína sobre la síntesis de TNF-α.

Otro mecanismo intracelular que puede estar implicado en los efectos de la lidocaína es a través de la atenuación de la activación de la MAPK “p38”, que al igual que NF-κβ aumenta la expresión de variedad de citoquinas proinflamatorias, dicha atenuación puede ser secundaria a una disminución de las concentraciones de calcio intracelular como lo demuestran Su y cols. en cultivos celulares (Su et al. 2014).

La lidocaína es capaz de disminuir la quimiotaxis de monocitos y puede lograr este efecto a través de una disminución en los niveles de MCP-1 (Bohlinger et al. 1996). La MCP-1 no solo actúa como quimiotáctico de monocitos, sino además es un conocido activador de sus funciones, que aumenta la síntesis y liberación de diversas citoquinas en el proceso inflamatorio (Ng et al. 2007).

En resumen, la lidocaína no solo es capaz de disminuir la quimiotaxis de monocitos sino también su activación todo lo cual contribuirá a la menor formación de TNF-α.

En este trabajo también hemos observado un aumento en los niveles de metaloproteinasas (MMPs) MMP-2 y MMP-9 como consecuencia de la cirugía. Al igual que lo que sucede con TNF-α, una elevación de las MMPs ha sido asociada al estrés quirúrgico. Diversos mecanismos pueden modificar la transcripción celular de las MMP entre ellos se encuentran la regulación por citoquinas, la señalización y factores nucleares (Iizasa et al. 1999). Entre las citoquinas capaces de aumentar la transcripción de MMPs encontramos al TNF-α y la IL-1, mientras que entre las vías de señalización intracelular se encuentran la MAPK “p38” y el NF-κβ (Soccal et al. 2004). Los niveles de MMP fueron disminuidos por la administración IV de lidocaína, lo cual podría contribuir a su efecto protector frente al daño pulmonar.

Numerosas citoquinas pro-inflamatorias, entre las cuales destaca el TNF-α, pueden activar la expresión de moléculas quimiotácticas (como MCP-1, monocyte chemoattractant

protein-1) o de moléculas de adhesión (como ICAM, intercellular adhesion molecule o VCAM, vascular cell adhesión molecule) las cuales atraen a los monocitos y a los neutrófilos hacia el tejido dañado. Una vez activados, tantos los monocitos como los neutrófilos, perpetúan y amplifican la respuesta inflamatoria contribuyendo en incrementar el daño inflamatorio. En nuestros trabajos hemos observado como en el daño por I/R pulmonar debido a trasplante lobular, hay un incremento tanto de los factores quimiotácticos, como de las moléculas de adhesión lo cual hace que se observe, a nivel pulmonar un incremento de los neutrófilos activados determinado mediante los niveles de mieloperoxidasa (MPO). En este caso también hemos observado que tanto la administración de lidocaína como el precondicionamiento, isquémico o anestésico, resultan eficaces en reducir estas alteraciones.

Todas las alteraciones inflamatorias descritas pueden resultar en un edema pulmonar, como ya se había comprobado en estudios previos de nuestro grupo (Simon Adiego et al. 2011, Casanova et al. 2011). Los tratamientos analizados, al reducir las alteraciones inflamatorias, reducen también el edema pulmonar lo cual podría implicar una significativa mejoría desde el punto de vista clínico, reduciendo la mortalidad post-trasplante y la aparición de daño pulmonar agudo (Acute lung injury, ALI).

Como ya se ha comentado anteriormente, la respuesta inflamatoria y el aumento del estrés oxidativo activados en los procesos de I/R pulmonar inducen edema pulmonar. Estudios recientes han propuesto que la formación del edema tras la I/R no se deba únicamente a un incrementado proceso inflamatorio, sino que también puede jugar un papel importante la pérdida de integridad del glicocálix endotelial (Chappell et al. 2009).

La pérdida de integridad del glicocálix causa un daño en las uniones intercelulares y una pérdida de función de la barriera endotelial lo cual concurre en la formación del edema pulmonar. De acuerdo con esto, en nuestro trabajo hemos observado una disminución de los niveles de heparán sulfato y sindecan-1, ambos marcadores de glicocálix, en pulmón. Por el contrario, los niveles plasmáticos de estos marcadores aumentaron. Estos resultados, indican una pérdida de integridad del glicocálix pulmonar secundaria a la cirugía de resección pulmonar. Además, la degradación del glicocálix se observó tras la reperfusión, lo cual sugiere que el daño al glicocálix podría contribuir en la creación del edema observado en nuestro modelo experimental. Estas alteraciones del glicocálix fueron evidentes no solo nivel local sinó también a nivel sistémico, observándose un incremento plasmático de los niveles de syndecan-1 y heparan sulfato tras el daño por I/R. La posibilidad de identificar este daño de manera poco invasiva podría tener relevancia para una posible aplicación clínica ya que nos permitiría monitorizar, en el paciente hospitalizado, no solamente el proceso inflamatorio, sino también alteraciones del endotelio.Estos resultados por lo tanto sugieren que la perdida de integridad del glicocálix juega un papel fundamental en la patogénesis del IRI. Sin embargo, nuestro modelo experimental no permite determinar cuál sea la relación de causa-consecuencia entre el daño del glicocálix y las alteraciones inflamatorias observadas en el pulmón tras la I/R. Ulteriores estudios serían necesarios para poder determinar con más claridad si el daño del glicocálix es causa y/o consecuencia de las alteraciones inflamatorias aunque, en nuestra opinión, podría ser tanto una cosa como la otra ya que el daño por I/R causa una serie de alteraciones que se retroalimentan y se activan mutuamente en un mecanismo de feedback positivo.

Por otro lado, el daño al glicocálix, fue acompañado de una mayor expresión de moléculas de adhesión y de un incremento dela actividad MPO, sugiriendo que la infiltración y activación de los neutrófilos podría jugar un papel importante en este proceso.

La administración de lidocaína bloqueó el efecto de la I/R sobre la perdida de integridad a cargo del glicocálix, tanto a nivel local como sistémico. Además, también se observó una menor expresión de las moléculas de adhesión y de la actividad de MPO en los animales a los que se administró lidocaína. Sin embargo, el mecanismo de acción por el que la lidocaína ejerce este efecto protector sobre el glicocálix, no está totalmente definido.

Recientemente, diferentes estudios han sugerido que los miRNAs podrían contribuir a la lesión por isquemia-reperfusión mediante la alteración de la expresión de diversos elementos clave en la supervivencia celular y la apoptosis (Bartel 2004, Ambros, Chen 2007). Los microRNAs son reguladores post-transcripcionales implicados prácticamente en todos los procesos celulares. Son reguladores finos de la información genética y crecientes evidencias ha demostrado que están implicados en los mecanismos fisiopatológicos subyacentes a numerosas de enfermedades.

Numerosos miRNAs han sido implicados en distintas enfermedades pulmonares, incluyendo patologías asociadas a procesos inflamatorios (Xie et al. 2012) y se ha descrito que alteraciones en la expresión de determinados miRNAs podrían participar en la regulación del proceso inflamatorio y la reparación tisular en la lesión pulmonar aguda (LPA)/SDRA (Williams et al. 2007, Angulo et al. 2012). Por otro lado, diferentes

investigadores han evidenciado la implicación de los miRNA en la respuesta inmune, innata y adquirida, implicada en el daño por I/R (Weiss et al. 2012). Por ello, hemos investigado un posible papel de algunos miRNAs en la fisiopatología de la I/R pulmonar.

Para seleccionar los miRNAs estudiados, nos hemos centrado en los miRNAs que habían sido previamente descritos en relación con el daño secundario a la isquemia-reperfusión, inflamación y/o patologías pulmonares (Tomankova et al. 2010, Oglesby et al. 2010, Weiss et al. 2012, Mas et al. 2013, Sessa, Hata 2013). Sin embargo, no todos los miRNAs descritos previamente en relación con estas patologías se han investigado en este estudio. Tras una exhaustiva revisión, elegimos, entre los que se relacionan con dichas patologías, aquellos miRNAs disponibles para cerdos.

Hemos observado que algunos miRNAs participaron en la primera fase de reperfusión (miR- 142-5p, miR-152, miR-155 and miR-223) mientras que en el caso de otros miRNAs (miR-21, miR-107, miR-126, miR-145, miR-146a, miR-182, miR-192 and let-7d) solo se modificó su expresión tras 60 minutos post reperfusión, indicando una activación más tardía. Estos resultados sugieren que los miRNAs podrían jugar un papel modulador de la respuesta inflamatoria en ambas fases, temprana y tardía, de reperfusión.

La respuesta inflamatoria juega un papel fundamental en el daño secundario a la I/R y ha sido descrito que varios miRNAs regulan o son regulados por ella (Sethu, Melendez 2011). La regulación positiva de miR-126 se ha correlacionado con la regulación de TOM1, que participa en la vía de señalización de las citoquinas proinflamatorias, IL-1β y TNF-α, en la fibrosis quística (Liu et al. 2010). Por otro lado, la desregulación de miR-155 y miR-223 indujo un aumento de la respuesta inflamatoria pulmonar en diferentes modelos experimentales (Park, Peter 2008). Además, se ha observado que miR-21 y miR-223 pueden promover la diferenciación y activación de los granulocitos (Roy, Sen 2011).

La infiltración y activación de neutrófilos es un componente importante del daño pulmonar secundario a la I/R (de Perrot et al. 2003). De acuerdo con esto, hemos observado un aumento de la actividad de MPO en pulmón, a los 30 y 60 minutos post reperfusión. Esto podría indicar una posible participación de los neutrófilos en el incremento de algunos miRNAs como miR-21 y miR-223 (leukocyte-derived miRNAs).

Por otro lado, miR-21, miR-145, miR-146a y miR-155, son regulados positivamente por NFκB y/o por citoquinas proinflamatorias como el TNF-α o la IL-1β (Taganov et al. 2006, O'Connell et al. 2007, Lorente-Cebrian et al. 2014, Xu et al. 2014). Sin embargo, sus efectos en la respuesta inflamatoria son contradictorios. Por una parte, ha sido descrito que miR-155 tiene efectos proinflamatorios (O'Connell et al. 2009, Pedersen et al. 2009, Costinean et al. 2009) mientras que miR-146a parece tener efectos anti inflamatorios (Boldin et al. 2011, Zhao et al. 2013). En nuestro estudio, hemos observado un aumento de la expresión de todos los miRNAs citados, sugiriendo su posible implicación en la respuesta inflamatoria secundaria a la I/R pulmonar. Sin embargo, dado que algunos de ellos tienen efectos pro-inflamatorios, mientras que otros tienen efectos antiinflamatorios y algunos, como miR-145 puede ser pro- (Yang et al. 2013) o anti-inflamatorios (Dharap et al. 2009), consideramos que investigaciones posteriores podrían aclarar el papel jugado por cada uno de los miRNAs lo cual permitiría una mejor comprensión de cómo múltiples miRNAs colaboran para equilibrar adecuadamente la respuesta inflamatoria a la I/R.

Por otro lado, el papel de los miRNAs en la regulación de la apoptosis es actualmente objeto de investigación intensiva. Estudios recientes han demostrado que la sobreexpresión of miR-145 (Dharap et al. 2009, Yang et al. 2013) y miR-192 (Feng et al.

2011) pueden inducir la apoptosis. De acuerdo con esto, hemos observado que el aumento de la expresión de miR-145 y miR-192 iba acompañado de un aumento de los niveles de marcadores proapoptóticos, sugiriendo que estos miRNAs podrían participar en la inducción de la apoptosis en respuesta a la I/R.

Estudios anteriores habían observado una disminución de la expresión de miR-16 (Cai et al. 2012) y miR-127 (Xie et al. 2012) en la lesión pulmonar aguda y el edema pulmonar (Tamarapu Parthasarathy et al. 2012). Sin embargo, en nuestro estudio, no se observó ningún cambio significativo en la expresión de miR-16 ni de miR-127, sugiriendo que estos miRNAs no parecen jugar un papel regulador en la patogénesis del daño pulmonar secundario a la I/R.

Las propiedades antiinflamatorias de la lidocaína ya han sido descritas. Sin embargo sus posibles efectos sobre la expresión de los miRNAs son poco conocidos. En estudios *in vitro* se ha observado que la lidocaína modificaba la expresión de miRNAs en células madre procedentes de tejido adiposo. Sin embargo, este efecto fue evidente solo en un número limitado (Sung et al. 2012). En nuestro estudio, la administración IV de lidocaína bloqueó de forma significativa el efecto de la I/R sobre la expresión de todos los miRNAs investigados. Al mismo tiempo, la lidocaína redujo la expresión de mediadores pro-inflamatorios y pro-apoptóticos. Estos resultados apoyarían la hipótesis de un posible papel modulador de los miRNAs en las respuestas inflamatorias y de apoptosis secundarias a la I/R pulmonar. Sin embargo, son necesarios estudios posteriores que permitan clarificar la posible relación entre los cambios en la expresión de miRNAs expresión y los efectos antiinflamatorios de la lidocaína.

Como ya se ha indicado anteriormente, durante la cirugía torácica, la OLV es una técnica ampliamente utilizada para facilitar el trabajo de los cirujanos durante las

intervenciones torácicas que permite aislar un pulmón de la ventilación mecánica. Esto condiciona un colapso de ese pulmón que permite trabajar en ese hemitórax y la necesidad de mantener una adecuada ventilación del paciente con el pulmón contralateral. Así, la ventilación unipulmonar provoca intensos cambios fisiopatológicos en la ventilación y la perfusión pulmonar. En este aspecto, se ha sugerido que la ventilación unipulmonar per se puede inducir una respuesta proinflamatoria, incluyendo liberación de citoquinas y migración de leucocitos en el pulmón ventilado (Zingg et al. 2010). De hecho, se han observado respuestas inflamatorias de carácter agudo en todos los contextos quirúrgicos que implican OLV, cuantitativamente superiores a las observadas en otras cirugías que conllevan ventilación bipulmonar (Sakamoto et al. 1994, Grichnik, D'Amico 2004).

En este trabajo, y con objeto de clarificar la contribución de la OLV al daño secundario a la cirugía pulmonar, hemos desarrollado también un modelo de ventilación unipulmonar (OLV) en el que tanto el tiempo de isquemia como el daño quirúrgico eran inferiores a los modelos de autotrasplante precedentemente investigados por nuestro grupo. Gracias a este modelo hemos podido observar una fuerte alteración de la respuesta inflamatoria tanto local como sistémica debida a la OLV. Observamos además que estas alteraciones eran evidentes desde tiempos de reperfusión cortos hasta las 24 horas post-cirugía. Además, hemos observado, que no solamente se ve afectado el pulmón sometido a I/R, sino que también son evidentes alteraciones en el pulmón ventilado con OLV, sugiriendo una influencia importante de este procedimiento ventilatorio en la respuesta inflamatoria y daño pulmonar secundario asociado al procedimiento quirúrgico. La OLV podría desencadenar un proceso inflamatorio por sí misma, lo cual ya había sido observado

en estudios previos (Baudouin 2003, Cree et al. 2004). Este aumento se asocia a la presencia de edema pulmonar, lo cual refleja el daño pulmonar que se produce como consecuencia de la respuesta inflamatoria y estaría de acuerdo con estudios previos que ya habían descrito una respuesta molecular similar (Baudouin 2003).

Como ya se ha indicado anteriormente, se ha sugerido que la apoptosis podría tener un papel central y clave en el daño pulmonar agudo, y probablemente su inhibición tendría un papel protector en este proceso inflamatorio (Dosreis et al. 2004). De acuerdo con ello, en nuestros estudios hemos observado un incremento de los mediadores por-apoptóticos en respuesta al daño por I/R. Esta activación de la apoptosis se produce de forma más acusada en el pulmón no ventilado durante la resección pulmonar, resultado concordante con los datos respecto a los parámetros inflamatorios. Estos resultados de nuevo confirman estudios previos de otros autores (Gothard 2006, Zingg et al. 2010), describiendo que la OLV *per se* causa daño a nivel pulmonar. Por ello, consideramos que, en futuros estudios, podría resultar interesante el desarrollo de un modelo experimental que nos permitiese diferenciar con más precisión el daño quirúrgico, el daño isquémico y el daño debido a la OLV. Como ya habíamos descrito anteriormente, en este modelo también, la lidocaína redujo la expresión de mediadores pro-inflamatorios y pro-apoptóticos demostrando de esta manera una atenuación del daño causado por isquemia reperfusión.

También hemos podido observar en nuestro modelo un aumento en los niveles de metalloproteinasas (MMPs). Al igual que lo que sucede con las citoquinas proinflamatorias, una elevación de las MMPs ha sido asociada al estrés quirúrgico, por lo que en cirugía mínimamente invasiva se observan valores sistémicos inferiores al compararla con la cirugía abierta (Ng et al. 2007).

Diversos mecanismos pueden modificar la transcripción celular de las MMPs, entre ellos se encuentran la regulación por citoquinas y factores de transcripción nuclear (Huang et al. 2009). Entre las citoquinas capaces de aumentar la transcripción de MMPs encontramos al TNFα y la IL-1, mientras que entre las vías de señalización intracelular se encuentran la MAPK "p38" y el NF-κβ (Nakamoto et al. 2000). Otros autores han demostrado una elevación de MMP-2 y MMP-9 secundaria a un daño por isquemia reperfusión en tejido pulmonar (Soccal et al. 2004, Yano et al. 2001) así como en otros tejidos (Cheung et al. 2000), relacionando el aumento de MMPs observado tras la agresión quirúrgica con la aparición de complicaciones postoperatorias.

En la literatura no hay estudios cuyo objetivo sea evaluar el efecto de los anestésicos locales sobre la producción de MMPs, sin embargo, como ya se mencionó entre los mecanismos implicados en la activación de la transcripción de las MMPs, encontramos la liberación de citoquinas proinflamatorias y vías de señalización intracelular sobre las cuales la lidocaína puede ejercer un papel inhibitorio lo cual podría contribuir a su efecto protector frente al daño pulmonar secundario a I/R.

En resumen, hemos observado que la respuesta inflamatoria y apoptótica asociada a la cirugía de resección pulmonar se produce a nivel bilateral, aunque de forma asimétrica. La OLV *per se* tiene una influencia en la activación de los procesos pro-inflamatorios y pro apoptóticos en ambos pulmones que es detectable 24 horas post-cirugía. Esto concuerda con otros resultados previos que relacionaban la lesión pulmonar por isquemia-reperfusión, condicionada por la OLV, con una activación de la inflamación y apoptosis (Ng et al. 2005).

Parece claro que la OLV es un factor clave que condiciona el grado de daño pulmonar postoperatorio en la CRP. Aquellas técnicas encaminadas a atenuar la agresión que supone esta técnica de ventilación (limitación de presión de ventilación, restricción de fluidos, acortar duración de la OLV…) podría por lo tanto ayudar a atenuar el grado de daño pulmonar postoperatorio de los pacientes sometidos a cirugías torácicas que impliquen este tipo de procedimiento ventilatorio.

Por otro lado, poder detectar alteraciones inflamatorias 24 horas después de la cirugía en muestras plasmática podría resultar útil para una futura aplicación clínica ya que nos permitiría evaluar de manera poco invasiva el progreso del paciente.

CONCLUSIONES

"No temas a las dificultades: lo mejor surge de ellas."

- *R. Levi Montalcini*

Exponemos a continuación las conclusiones más significativas:

1. La cirugía de resección pulmonar indujo un aumento significativo de los niveles de mediadores inflamatorios y/o apoptóticos, tanto a nivel local como sistémico (Artículos I, II, III, IV, V, VI y VII)

2. Estos cambios podrían ser secundarios tanto a la I/R propia de la cirugía torácica como a la ventilación unipulmonar (OLV), técnica ampliamente utilizada durante las intervenciones torácicas (Articulo III)

3. El aumento de los niveles de mediadores proinflamatorios fue acompañado por modificaciones en el perfil de expresión de microRNAs y alteraciones en la integridad del glicocálix (Artículos IV y V)

4. La modulación anestésica con sevoflurano o lidocaína, disminuyó significativamente estos efectos tanto a nivel local como sistémico. (Artículos II, III, IV, V, VI y VII)

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ANEXOS

ARTÍCULO I

Ischaemic preconditioning prevents the liver inflammatory response to lung ischaemia/reperfusion in a swine lung autotransplant model.

Luis Huerta, Lisa Rancan, Carlos Simón, Jesús Isea, Eduardo Vidaurre, Elena Vara, Ignacio Garutti and Federico González-Aragoneses

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ORIGINAL ARTICLE

EXPERIMENTAL

Ischaemic preconditioning prevents the liver inflammatory response to lung ischaemia/reperfusion in a swine lung autotransplant model†

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Abstract

OBJECTIVES: Lung ischaemia/reperfusion (IR) induces a systemic inflammatory response that causes damage to remote organs. The liver is particularly sensitive to circulating inflammatory mediators that occur after IR of remote organs. Recently, remote ischaemic preconditioning has been proposed as a surgical tool to protect several organs from IR. The present study was designed to investigate a possible protective effect of lung ischaemic preconditioning (IP) against the liver inflammatory response to lung IR.

METHODS: Two groups [IP and control (CON)] of 10 Large White pigs underwent lung autotransplants (left pneumonectomy, ex situ cranial lobectomy and caudal lobe reimplantation). Before pneumonectomy was performed in the study group, IP was induced with two 5-min cycles of left pulmonary arterial occlusion and a 5-min interval of reperfusion between the two occlusions. Five animals underwent sham surgery. Liver biopsies were obtained during surgery at (i) prepneumonectomy, (ii) prereperfusion, (iii) 10 min after reperfusion of the implanted lobe and (iv) 30 min after reperfusion. The expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-10 and inducible form of nitric oxide synthase (iNOS) was analysed by western blotting. The expression of mRNA for TNF-α, IL1, IL-10, monocyte chemoattractant protein-1 (MCP-1), nuclear factor kappa beta and iNOS was analysed by reverse transcription–polymerase chain reaction. Caspase-3 activity was determined by enzyme-linked immunosorbent assay. Non-parametric tests were used to compare differences between and within groups.

RESULTS: Lung IR markedly increased expression of TNF-α ($P = 0.0051$) and IL-1 ($P = 0.0051$) and caspase-3 activity ($P = 0.0043$) in the CON group compared with the prepneumonectomy levels. A decrease of IL-10 mRNA expression was observed in the CON group after lung reperfusion. In the IP group, TNF-α ($P = 0.0011$) and IL-1 ($P = 0.0001$) expression and caspase-3 activity ($P < 0.0009$) were lower after reperfusion than in the CON group. IP caused reversion of the observed decrease of IL-10 mRNA expression ($P = 0.016$) induced in liver tissue by lung IR. Lung IR markedly increased the expression of mRNA MCP-1 after 10 min ($P = 0.0051$) and 30 min ($P = 0.0051$) of reperfusion. These increases were not observed in the IP or sham groups.

CONCLUSIONS: IP prevented liver injury induced by lung IR through the reduction of proinflammatory cytokines and hepatocyte apoptosis.

Keywords: Ischaemia/reperfusion injury • Ischaemic preconditioning • Lung • Liver

INTRODUCTION

Lung ischaemia/reperfusion (IR) induces a local inflammatory response in pulmonary tissue characterized by non-specific alveolar damage, lung oedema and hypoxaemia. Many different mediators have been implicated in the pathogenesis of IR lung injury, and several authors have shown that the increase in lung biomarkers is related to postoperative pulmonary complications and poor postoperative outcome [1, 2]. Lung IR may also induce a systemic inflammatory response that causes damage to remote organs that is more detrimental than its local effects. The liver is particularly sensitive to circulating inflammatory mediators that occur after IR of remote organs, such as the kidney [3–5], gut [6] and skeletal muscle [7, 8]. The findings from one experimental study in rabbits suggest that pulmonary IR induces liver injury characterized by activated neutrophil sequestration and the release of significant amounts of reactive oxygen species [9]. Thus, once IR lung injury occurs and the liver suffers the consequences, treatment must focus on both local and remote responses. Furthermore, prevention strategies against lung IR injury should address their beneficial effects on both lung and liver injuries.

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Direct ischaemic preconditioning (IP) has been proven to protect several organs from IR injury. Recently, remote ischaemic preconditioning (RIPC), defined as IP by repetitive ischaemic episodes in an organ that is remote from the organ to be protected, has been proposed as a surgical tool to prevent cardiac, hepatic or brain ischaemia. In those studies, limb and liver ischaemia have been the main sites of preconditioning stimuli. The authors have previously shown that, in a swine model of lung autotransplantation, IP attenuated lung IR injury by preventing increases in lipid peroxidation metabolites, leukocyte activation and proinflammatory cytokines in lung tissue [10]. However, little is known about the effects of IP on liver response to lung IR. The present experimental study was designed to investigate a possible protective effect of IP against the liver inflammatory response to lung IR.

MATERIALS AND METHODS

This study was approved by the institution's Research and Animal Experimentation Committee, and all of the animals received humane care in compliance with the European Convention on Animal Care.

Animal model and study groups

Twenty Large White pigs weighing 35–50 kg underwent an orthotopic left lung autotransplantation (left pneumonectomy, ex situ cranial lobectomy and left caudal lobe reimplantation) with a subsequent 30-min graft reperfusion. The animals were grouped by random numbers (Microsoft Excel 2003) to receive lung autotransplantation without IP [control (CON) group, $n = 10$] or with an IP procedure (IP group, $n = 10$). In addition, five animals underwent sham surgery (SHAM group).

Anaesthesia and surgical protocols

The anaesthesia protocol and the surgical technique for this lung autotransplant model have previously been described in detail [10, 11]. Briefly, premedication was performed using intramuscular ketamine 10 mg/kg (Ketoral; Parker Davis). Once in the operating room, pulsioxymetry and electrocardiographic monitoring were performed. Anaesthesia induction was achieved with propofol 4 mg/kg (Diprivan; Fresenius K), fentanyl 3 μg/kg (Fentanest; Kern Pharma) and atracurium 0.6 mg/kg (Tracrium; GlaxoSmithKline). Orotracheal intubation was performed, and mechanical ventilation was provided with a Drager SA-1 ventilator (tidal volume 8 ml/kg, respiratory rate 12–15 rpm and inspiratory/expiratory ratio of 1:2 to maintain PaCO2 in the range of 35–40 mmHg). FiO2 was maintained at 1 throughout the procedure. Intraoperative crystalloid infusion was maintained at 6–8 ml/kg/h. Anaesthesia was maintained with propofol in continuous perfusion (8–10 mg/kg/h) throughout the experiment. The supplemental doses of fentanyl and atracurium were used when required. A surgical tracheotomy was performed, the orotracheal tube was removed and a 6-mm cuffed tube was inserted into the trachea through the tracheotomy. A 7-F pulmonary artery catheter (Edwards Lifesciences) was introduced through the femoral vein. A 7-F femoral artery catheter was used for blood pressure monitoring and blood sampling.

A left thoracotomy was carried out by means of a fourth or fifth rib resection followed by a 5- to 6-cm subxiphoid midline laparotomy to allow liver biopsies to be performed. To perform left pneumonectomy, the pulmonary artery, cranial vein, caudal vein and main left bronchus were progressively dissected. Two-lung ventilation was maintained until the pulmonary vessels were dissected, the main left bronchus was sectioned and the endotracheal tube was placed into the right bronchus. Just before the pneumonectomy was completed, a bolus of intravenous heparin 300 IU/kg (MaynePharma Spain, S.L.) was administered to prevent thrombosis in the clamped pulmonary artery. Next, on the back table, the left lung was perfused through the pulmonary artery and veins with University of Wisconsin solution at 10–15°C until a clear effluent from the pulmonary vessels was observed. A cranial lobectomy was carried out, and the caudal left lobe was then implanted back into the animal by performing a bronchus-to-bronchus anastomosis, a pulmonary artery-to-pulmonary artery anastomosis and an inferior vein-to-left atrium anastomosis. Graft reperfusion was performed initially in a retrograde direction by unclamping the left atrium, and then the endobronchial tube was pulled back into the trachea, enabling two-lung ventilation. Anastomotic patency of the atrial anastomosis was determined by active bleeding through the open pulmonary artery anastomosis. The left pulmonary artery was then unclamped, and blood flow was maintained for 30 min. At the end of the experiment, the animal was euthanized with a potassium chloride injection under deep anaesthesia. In the experimental group, 5 min before the dissected pulmonary vessels and the main left bronchus were sectioned, two separate 5-min left pulmonary arterial clamping attempts were carried out, with a 5-min interval reperfusion between the two occlusions (Fig. 1). The animals in the SHAM group underwent the same protocol, including thoracotomy, except for lung resection and one-lung ventilation (OLV). In this group, no pulmonary artery clamping was performed at any time.

Measurement and sampling time points

Baseline (B) haemodynamic and arterial blood gas measurements were performed 30 min after the thoracotomy, with the animal under two-lung ventilation. Subsequently, haemodynamic and arterial gas measurements and liver biopsies were performed at the following time points (Fig. 1): prepneumonectomy (PPn)—before completing the pneumonectomy and with the animal under OLV; prereperfusion (PRp)—before reperfusion and ventilation of the reimplanted left caudal lobe; 10-min postreperfusion (Rp-10′)—10 min after the reperfusion of the reimplanted lobe and 30-min postreperfusion (Rp-30′)—30 min after the reperfusion of the reimplanted lobe. In the experimental group, PPn measurements and biopsies were performed after the IP manoeuvres. In the sham experiments, time points were taken as follows: PPn—120 min after thoracotomy; PRp—120 min after PPn; Rp-10′—10 min after PRp and Rp-30′—30 min after PRp.

Haemodynamic measurements

Additional variables were determined at the above-mentioned time points. A femoral artery catheter was used to record the mean blood pressure. The pulmonary-artery catheter recorded the pulmonary artery mean pressure. In addition, the cardiac

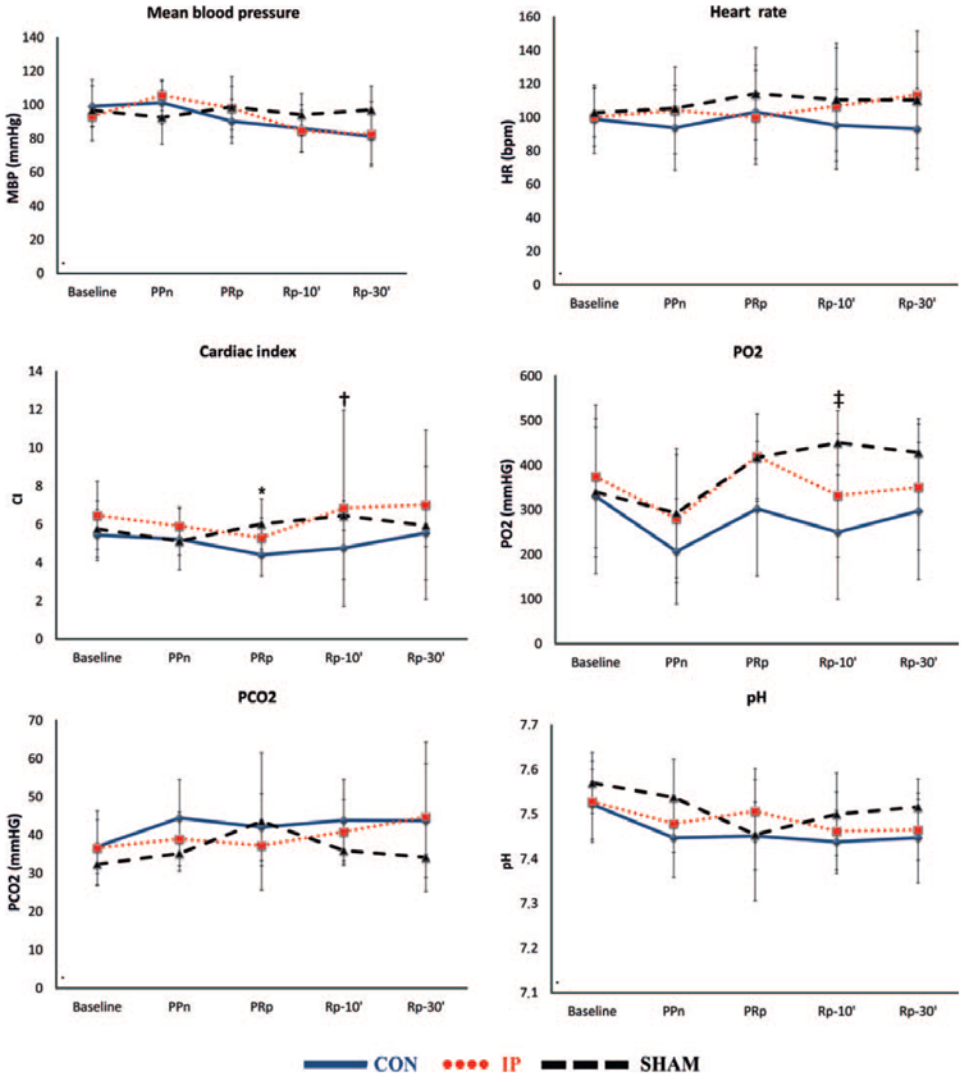

Figure 1: Haemodynamic and gasometric data. The lines show haemodynamic and gasometric values (mean ± SD) throughout the experiment in each group. MBP: mean blood pressure; HR: heart rate; CI: cardiac index; PO2: systemic partial pressure of oxygen; PCO2: systemic partial pressure of carbon dioxide; CON: control group; IP: ischaemic preconditioning group; SHAM: sham group; B: basal; PPn: prepneumonectomy; PRp: prereperfusion; Rp-10′: 10-min postreperfusion; Rp-30′: 30-min postreperfusion. *$P = 0.042$, CON vs SHAM group; †$P = 0.049$, CON vs SHAM group; ‡$P = 0.019$, CON vs SHAM group.

output monitor (Edwards Lifesciences) and thermodilution technique were used at the mentioned time points to record the cardiac index (CI).

Blood gas measurements

Arterial blood gas analyses were performed at the previously mentioned time points. In addition, blood gas samples were taken by puncturing the pulmonary vein of the reimplanted lobe at 10 and 30 min after reperfusion.

Biochemical studies in liver tissue

Liver tissue biopsies were performed for biochemical studies. Every liver sample was placed in a cryotube, flash frozen in liquid nitrogen and stored at −80°C until biochemical analysis.

RNA isolation and reverse transcription–polymerase chain reaction

A TRI Reagent Kit (Molecular Research Centre, Inc., Cincinnati, OH, USA) was used according to the manufacturer's protocol to isolate RNA from swine liver samples following the method described by Chomczynski and Sacchi [12]. The purity of the RNA was estimated by analysis using 1.5% agarose gel electrophoresis, and the RNA concentrations were determined by spectrophotometry (260 nm). The Reverse Transcription System (Promega, Madison, WI, USA) and a pd(N)6 random hexamer were used to perform reverse transcription of 2 μg of RNA for cDNA synthesis. An Applied Biosystems 7300 apparatus with the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 300 nM concentrations of specific primers were used to perform reverse transcription–polymerase chain reaction (RT–PCR; Table 1). RT–PCR amplifications were performed as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. For normalization of cDNA loading in the PCR, amplification of 18S rRNA for every sample was used. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression [13].

Preparation of liver homogenates and determination of caspase-3

Frozen organ samples were weighed and transferred to 50-ml polypropylene tubes (Falcon; Becton Dickinson, Lincoln Park, NJ, USA) containing lysis buffer (4°C) at a ratio of 10 ml buffer/1 g of wet tissue. The lysis buffer consisted of 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Company), 1 mg/ml pepstatin A (Sigma Chemical Company), aprotinin (Sigma Chemical Company) and leupeptin (Sigma Chemical Company) in 1× phosphate buffered saline solution of pH 7.2 (Biofluids, Rockville, MD, USA) containing 0.05% sodium azide (Sigma Chemical Company). The samples were homogenized for 30 s with an electrical homogenizer (Polytron; Brinkmann Instruments, Westminster, NY, USA) at maximum speed, and the tubes were immediately frozen in liquid nitrogen. The samples were homogenized three times for optimal processing. The homogenates were later thawed in a 37°C water bath and centrifuged at 119 000 g (1 h, 4°C) to separate cellular organelles. The supernatants were frozen at −80°C to allow the formation of macromolecular aggregates. After thawing at 4°C, the aggregates were pelleted at 3000 g (4°C), and the final organ homogenate volume was measured with a graduated pipette. The homogenates were stored at −80°C until they were assayed for the quantitative presence of cytokines. An enzyme-linked immunosorbent assay (ELISA) kit was used according to the manufacturer's instructions (Sigma Chemical Company) to measure caspase-3 in the liver homogenates collected from all groups of swine.

Table 1: Gene-specific primers used for RT–PCR

	Primers	Sequence (5′–3′)
18S	Forward	GGTGCATGGCCGTTCTTA
	Reverse	TCGTTCGTTATCGGAATTAACC
TNF-α	Forward	ATGAGAAGTTCCCAAATGGC
	Reverse	CTCCACTTGGTGGTTTGCTA
IL-1β	Forward	TGTGATGAAAGACGGCACAC
	Reverse	CTTCTTCTTTGGGTATTGTTTGG
IL-10	Forward	ACTGCACCCACTTCCCAGT
	Reverse	TTGTCCAGCTGGTCCTTTGT
iNOS	Forward	CTTTGCCACGGACGAGAC
	Reverse	TCATTGTACTCTGAGGGCTGAC
MCP-1	Forward	AGCATCCACGTGCTGTCTC
	Reverse	GATCATCTTGCCAGTGAATGAGT
NF-κB1	Forward	CAGCTCTTCTCAAAGCAGCA
	Reverse	TCCAGGTCATAGAGAGGCTCA

Western blot analysis

Western blots were used to measure the protein expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-1b, IL-10 and inducible form of nitric oxide synthase (iNOS). Briefly 50- to 60-mg liver samples was homogenizated with lysis buffer (ratio 20:250) and sonicated. Samples were then boiled with gel-loading buffer (0.100 M Tris–Cl; 4% sodium dodecyl sulphate; 20% glycerol; 0.1% bromophenol blue) (ratio 1:1). Protein concentration was determined by Bradford method. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis with 10% acrylamide gels was used to separate the total protein equivalents (30 mg) for each of the samples, which were transferred onto a nitrocellulose membrane in a semi-dry transfer system. The membrane was immediately placed into a blocking buffer containing 5% non-fat milk in 20 mM Tris, pH 7.5; 150 mM NaCl and 0.01% Tween-20. The blot was allowed to block at 37°C for 1 h. The membrane was incubated with rabbit polyclonal TNF-α (1:4000; BioGenesis), IL-1β (1:4000; BioGenesis), IL-10 (1:4000; BioGenesis) and iNOS (1:1000; BioGenesis) overnight at 4°C and then incubated in an anti-rabbit immunoglobulin G-horseradish peroxidase-conjugated antibody (1:2000). After washing with T-TBS (Tris-buffered saline with tween 20), the membranes were incubated with ECL Plus detection reagents (Amersham Life Science, Inc., Buckinghamshire, UK) and exposed to X-ray film. The films were scanned with a densitometer (BioRad GS 800) to determine the relative optical densities. Prestained protein markers were used for molecular weight determinations. Reproducibility within the assays was evaluated in three independent experiments. Each assay was performed with three replicates. The overall intra-assay coefficient of variation was <5%. Assay-to-assay reproducibility was evaluated in three independent experiments. The overall interassay coefficient of variation was <6%.

Statistical analysis

The data are expressed as the mean ± standard deviation (SD). Non-parametric tests were used. Accordingly, a Mann–Whitney *U*-test was applied to establish differences between the analysed groups. In addition, a Wilcoxon test for paired data was used to study the evolution of the intragroup values. The level of statistical significance was set at $P < 0.05$. The SPSS version 14.0 statistical package was used in this study.

RESULTS

There were no differences among the SHAM, CON and IP groups in terms of animal weight (40 ± 11, 47 ± 14 and 38 ± 14

kg, respectively) or duration of the entire procedure (291 ± 10, 287 ± 35 and 308 ± 38 min, respectively). There were no differences between the CON and IP groups in terms of lung ischaemia time (100 ± 17 vs 108 ± 10 min).

Haemodynamics and blood gas analysis

The haemodynamic and arterial gasometric values showed great stability. The only significant differences observed were in the CI at PRp and Rp-10′ and in PO2 at Rp-10′ between the SHAM and CON animals (Fig. 1).

Expression of mRNA for TNF-α, IL-1 and IL-10

TNF-α mRNA expression in liver tissue increased after ischaemia in the CON group ($P = 0.0093$, PRp vs PPn), but not in the SHAM or IP group (Fig. 2A). IL-1 mRNA expression increased after reperfusion in the CON ($P = 0.0069$, Rp-10′ vs PRp) and IP ($P = 0.046$, Rp-10′ vs PRp) livers, but not in the SHAM livers. After 30 min of lung reperfusion, the increase in IL-1 mRNA expression in liver tissue was significantly lower when IP was performed ($P = 0.00025$, CON vs IP) (Fig. 2B). Conversely, after lung reperfusion, a decrease in IL-10 mRNA expression was observed in the CON livers, but not in the IP or SHAM livers (Fig. 3).

Western blot analysis of TNF-α, IL-1 and IL-10

Lung IR markedly increased the expression of the proinflammatory cytokines TNF-α and IL-1 in the CON group livers at PRp, Rp-10′ and Rp-30′ when compared with the PPn levels. A similar increase in the expression of TNF-α was observed in the IP livers, but not in the SHAM livers (Fig. 2A). The increases in expression of TNF-α and IL1 in the CON group were higher than those of the IP group at PRp ($P = 0.0029$ and 0.0039, respectively), Rp-10′ ($P = 0.0015$ and 0.0001, respectively) and Rp-30′ ($P = 0.0011$ and 0.0001, respectively) (Fig. 2B). No significant differences in the anti-inflammatory cytokine IL-10 were observed (Fig. 3).

Expression of mRNA for MCP-1

Lung IR markedly increased the expression of mRNA monocyte chemoattractant protein-1 (MCP-1) in liver tissue at PRp ($P = 0.037$), Rp-10′ ($P = 0.0051$) and Rp-30′ ($P = 0.0051$) compared with the PPn levels. These increases were not observed in the IP or SHAM groups (Fig. 4).

Expression of iNOS, iNOS mRNA and NF-κB mRNA

No significant changes were found for iNOS or iNOS mRNA expression during the procedure. Similarly, no differences were found in the expression of nuclear factor kappa beta (NF-κB) mRNA among the three groups. A significant increase in NF-κB mRNA expression was observed at Rp-10′ compared with PPn in CON livers ($P = 0.0069$), but not in the SHAM or IP livers (Fig. 5).

ELISA quantification of caspase-3

Caspase-3 protein levels continuously increased throughout the procedure in all three groups (Fig. 6). This increase was higher in the IP than in the SHAM livers at Rp-30′ ($P = 0.016$), but was even higher in the CON than in the IP livers ($P = 0.009$, CON vs IP at PRp, Rp-10′ and Rp-30′).

DISCUSSION

It is well known that after some cardiac or lung surgeries, lung IR may cause pulmonary dysfunction that increases perioperative morbidity and mortality. Moreover, the events of IR injury trigger a systemic inflammatory response and multiple organ dysfunction syndrome that are potentially more detrimental than its local effects. Few studies have investigated the effects of IR lung injury on remote organs. Esme *et al.* [9] showed that pulmonary IR induced liver injury in rabbits. In that study, a 60-min I/R of lung tissue was enough to increase hepatic myeloperoxidase activity, reflecting recruitment of neutrophils into the liver. In addition, they also observed an increase in the markers of oxidative stress in liver tissue after lung IR. Similarly, in the present experiment, lung IR induced a remote hepatic inflammatory response as evidenced by the striking increase in expression of the proinflammatory cytokines TNF-α, IL-1 and MCP-1 in the livers of the control animals. We also observed that lung IR induced a progressive decrease in IL-10 mRNA expression in CON livers, reflecting an increasing imbalance between pro- and anti-inflammatory cytokine production. These results are consistent with reports by others that have suggested that IR of a distant organ, including the kidney [3, 5], gut [6] and limbs [14–16], may induce the release of cytokines and other inflammatory mediators by hepatic cells. The authors have previously shown in the same experimental model that lung IR induced an increase in proinflammatory cytokines and oxidative stress parameters in lung tissue [11], and we can speculate that lung IR injury may initiate a cascade of proinflammatory pathways that facilitates organ crosstalk and remote organ injury. The same may be said for the activation of apoptotic pathways. Some authors have found that IR can induce apoptosis in remote organs, including the heart [4], liver [17] and lungs [18–20]. McCarter *et al.* found an increase in liver apoptosis in a murine model of limb IR. They showed that caspase-3 activity, a hallmark indicator of apoptosis, increased 3-fold after 3 h of reperfusion. This increase in caspase-3 activity in liver tissue was followed by increased DNA fragmentation after 6 h of reperfusion [17]. These results are consistent with those of our study, where caspase-3 increased throughout the procedure in CON livers to nearly 1-fold higher than that in the SHAM group after 30 min of lung reperfusion. In light of these findings, it is conceivable that inflammatory mediators produced in the ischaemic lung circulate upon reperfusion and induce both inflammatory and apoptotic responses in the liver.

Direct IP has been proven to reduce experimental and clinical lung IR injuries, and we have previously shown that lung IP prevents an increase in oxidative stress, leukocyte activation and proinflammatory cytokines (TNF-α, IL-1 and MCP-1) in lung tissue during pulmonary IR [10]. The present study goes beyond our previous findings and demonstrates that lung IP reduced the inflammatory and apoptotic responses induced in the liver by

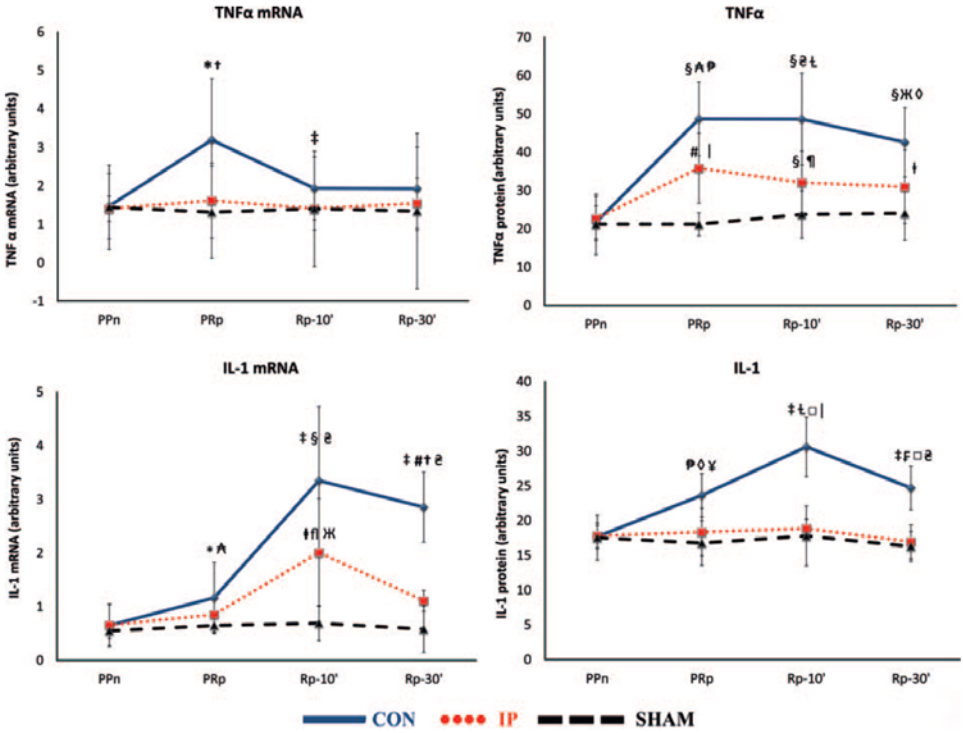

Figure 2: (**A**) The lines show the expression of TNF-α mRNA and TNF-α in liver tissue throughout the experiment in the experimental groups. CON: control group; IP: ischaemic preconditioning group; SHAM: sham group; PPn: prepneumonectomy; PRp: prereperfusion; Rp-10′: 10-min postreperfusion; Rp-30′: 30-min postreperfusion. *Intragroup analysis:* *$P = 0.0093$ vs PPn; †$P = 0.049$ vs PRp; §$P = 0.0051$ vs PPn; #$P = 0.0069$ vs PPn; ‖$P = 0.037$ vs PPn. *Intergroup analysis:* ‡$P = 0.047$, CON vs IP group; ^$P = 0.0029$, CON vs IP group; ℮$P = 0.0015$, CON vs IP group; $P = 0.0011$, CON vs IP group; ¥$P = 0.00066$, CON vs SHAM group; £$P = 0.0013$, CON vs SHAM group; ◊$P = 0.0027$, CON vs SHAM group; ǂ$P = 0.0047$, IP vs SHAM group; ¶$P = 0.04$, IP vs SHAM group. (**B**) The lines show the expression of IL-1 mRNA and IL-1 in liver tissue throughout the experiment in the experimental groups. *Intragroup analysis:* *$P = 0.049$ vs PPn; †$P = 0.0051$ vs PPn; §$P = 0.0069$ vs PRp; #$P = 0.013$ vs PRp; ‖$P = 0.028$ vs PPn; ¶ $P = 0.046$ vs PRp; ¥$P = 0.0069$ vs PPn; £$P = 0.0051$ vs PRp; ℮$P = 0.0051$ vs Rp-10′. *Intergroup analysis:* ‡$P = 0.00025$, CON vs IP group; ^$P = 0.028$, CON vs SHAM group; ℮$P = 0.00066$, CON vs SHAM group. $P = 0.0303$, IP vs SHAM group; ◊$P = 0.0039$, CON vs IP group; $P = 0.0001$, CON vs IP group; ǂ$P = 0.0047$, CON vs SHAM group; ǂ$P = 0.0013$, CON vs SHAM group.

lung IR injury. In light of these results, it is tempting to speculate that the benefits of IP on lung IR-induced systemic inflammatory response may reflect the protection of both the lungs and remote organs. In fact, RIPC has recently been proposed as a method of IR injury prevention. In RIPC, brief ischaemia of one organ has been shown to confer protection on distant organs without direct stress to the organ. Experimental studies have demonstrated that brief I/R of the limb, gut, mesentery or kidney reduces myocardial infarct size, and clinical research has shown that skeletal IP induces myocardial protection [21]. Two reports have shown that limb IP has a protective role against hepatic IR injury in rats [7, 8]. The underlying mechanisms and pathways of RIPC are not well established, and an overlap between the neurogenic and humoral pathways has been proposed [21]. Remote cytokine (including TNF-α [22, 23], IL-1 [22] and MCP-1[24]), NF-κB [25] and iNOS [25] modulation through these signalling pathways have been suggested as a potential mechanism for RIPC-induced protection. In our experiment, lung IP prevented the rise of TNF-α, IL-1 and MCP-1 induced by lung IR in liver tissue, although no differences in NF-κB mRNA or iNOS expression were found. However, the differences in the experimental models (hind limb RIPC in mice hearts) and measurement time points (2–24 h) used in our study and the cited research [25] could explain the discrepancies among the studies. Finally, in our investigation, lung IP prevented liver apoptosis after lung IR as reflected by the lower levels of caspase-3 protein in the hepatic tissue of the animals in which lung IP was performed. Similarly, others have shown that RIPC reduced apoptosis in mice livers after limb IR [17].

A considerable limitation of this work is the complex nature of the surgical procedure. Although the hepatic inflammatory and apoptotic responses seemed to be preferentially enhanced by lung reperfusion, increases in TNF-α, IL-1 and MCP-1 expression were observed in the CON livers before the beginning of pulmonary reperfusion. These findings suggest that, in this experimental model, the liver inflammatory response may be induced by both surgical stress and remote IR injury. However, IR and surgical stress inevitably occur in conjunction with these

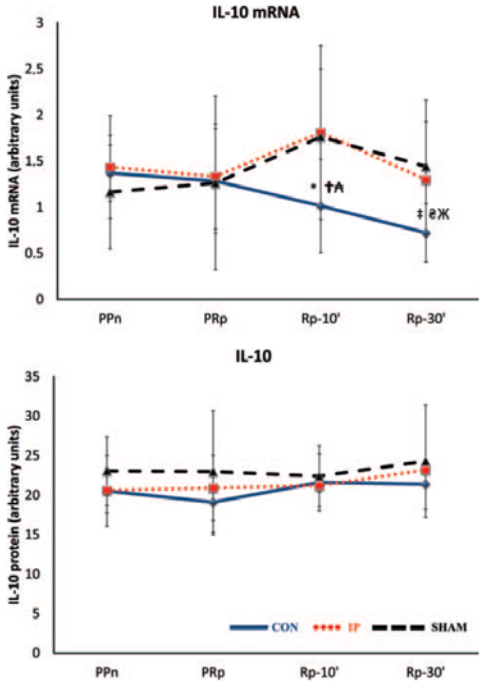

Figure 3: The lines show the expression of IL-10 mRNA and IL-10 in liver tissue throughout the experiment in the experimental groups. CON: control group; IP: ischaemic preconditioning group; SHAM: sham group; PPn: prepneumonectomy; PRp: prereperfusion; Rp-10′: 10-min postreperfusion; Rp-30′: 30-min postreperfusion. *Intragroup analysis*: *$P = 0.047$ vs PPn; †$P = 0.0051$ vs PPn. *Intergroup analysis*: †$P = 0.023$, CON vs IP group; ^$P = 0.028$, CON vs SHAM group; ‡$P = 0.016$, CON vs IP group; $P = 0.04$, CON vs SHAM group.

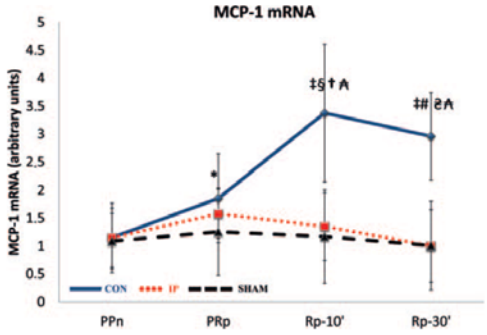

Figure 4: The lines show the expression of the chemokine MCP-1 mRNA in liver tissue throughout the experiment in the experimental groups. MCP-1: monocyte chemoattractant protein-1; CON: control group; IP: ischaemic preconditioning group; SHAM: sham group; PPn: prepneumonectomy; PRp: prereperfusion; Rp-10′: 10-min postreperfusion; Rp-30′: 30-min postreperfusion. *Intragroup analysis*: *$P = 0.037$ vs PPn; †$P = 0.0051$ vs PPn; §$P = 0.028$ vs PRp; #$P = 0.021$ vs PRp. *Intergroup analysis*: †$P = 0.00013$, CON vs IP group; ^$P = 0.0027$, CON vs SHAM group; ‡$P = 0.00008$, CON vs IP group.

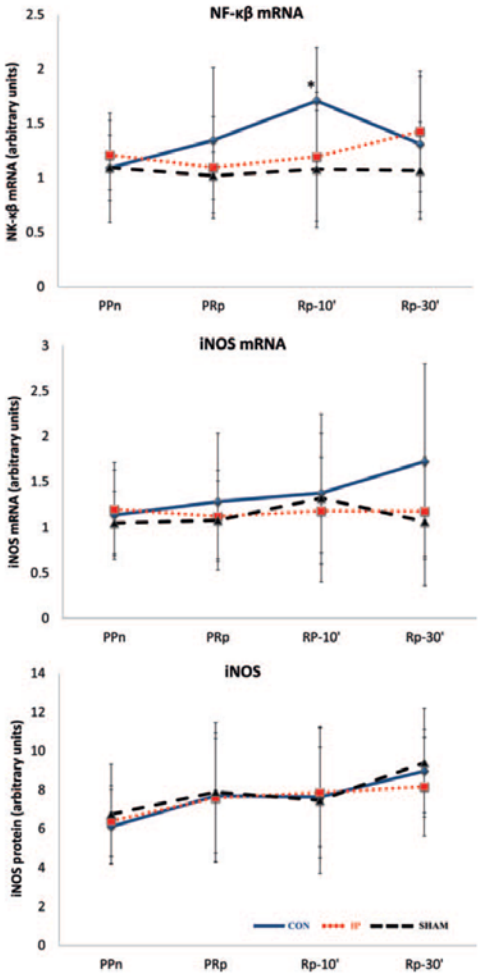

Figure 5: The lines show the expression of the transcription factor NF-κB, iNOS and iNOS mRNA in liver tissue throughout the experiment in the experimental groups. iNOS: inducible nitric oxide synthase; CON: control group; IP: ischaemic preconditioning group; SHAM: sham group; PPn: prepneumonectomy; PRp: prereperfusion; Rp-10′: 10-min postreperfusion; Rp-30′: 30-min postreperfusion. *$P = 0.0069$ vs PPn.

procedures, and there is no conclusive way to discern their respective impact on liver response.

In conclusion, the findings of this research have obvious relevance because the surgical protocol used, mimics clinical procedures (cardiopulmonary bypass, pulmonary thromboembolectomy, bronchovascular sleeve resection, lung transplantation) and because RIPC appeared to confer protection from lung IR injury. The clinical significance of pulmonary IR-induced remote organ dysfunction should be further studied to guide thoracic surgeons in the management of patients with remote organ dysfunction.

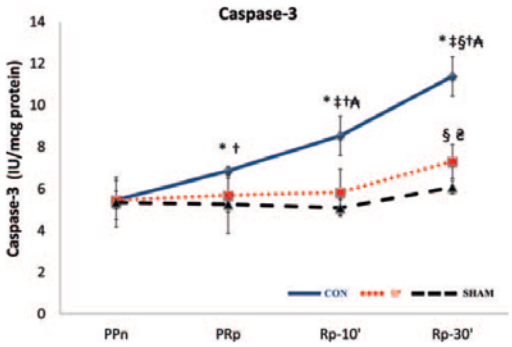

Figure 6: The lines show the expression of the caspase-3 concentration in liver tissue throughout the experiment in the experimental groups. CON: control group; IP: ischaemic preconditioning group; SHAM: sham group; PPn: prepneumonectomy; PRp: prereperfusion; Rp-10′: 10-min postreperfusion; Rp-30′: 30-min postreperfusion. *Intragroup analysis*: *$P = 0.043$ vs PPn; †$P = 0.043$ vs PRp; §$P = 0.043$ vs Rp-10′. *Intergroup analysis*: ‡$P = 0.009$, CON vs IP group; ^$P = 0.0079$, CON vs SHAM group; ²$P = 0.016$, IP vs SHAM group.

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Conflict of interest: none declared.

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APPENDIX. CONFERENCE DISCUSSION

Dr J. Fibla *(Barcelona, Spain)*: Do you think there might be a specific future clinical application of the results?

Dr Huerta: Yes. Until we find a molecular combination of mediators to perform pharmacological preconditioning, we think that ischemic preconditioning is a clinical tool to perform for protecting remote ischemic preconditioning from ischemia-reperfusion induced damage.

Dr Y. Colson *(Boston, MA, USA)*: Do you think that there are specific mediators that you could maybe block or change so that you wouldn't have to do the preconditioning clinically?

Dr Huerta: We think that the mediators that are implicated in ischemia-reperfusion are not well defined, and believe that the increase in inflammatory mediators may be induced by both the surgical stress and the remote ischemia-reperfusion injury.

ARTÍCULO II

Sevoflurane prevents liver inflammatory response induced by lung ischemia-reperfusion

Lisa Rancan, Luis Huerta, Gabriel Cusati, Iñaki Erquicia, Jesús Isea, Sergio D. Paredes, Cruz García, Ignacio Garutti, Carlos Simón, Elena Vara

Sevoflurane Prevents Liver Inflammatory Response Induced by Lung Ischemia-Reperfusion

Lisa Rancan,[1,5] Luis Huerta,[2] Gabriel Cusati,[3] Iñaki Erquicia,[3] Jesús Isea,[2] Sergio D. Paredes,[4] Cruz García,[1] Ignacio Garutti,[3] Carlos Simón,[2] and Elena Vara[1]

Background. Transplants cause ischemia-reperfusion (IR) injury that can affect distant organs. Liver is particularly sensitive to IR injury. The present randomized experimental study was designed to investigate a possible protective effect of sevoflurane against liver inflammatory response to lung IR in a lung upper lobe left autotransplant model.
Methods. Two groups (sevoflurane and control) of eight swines each were submitted to upper lobe left lung autotransplant. Hypnotic maintenance was performed with sevoflurane 3% or propofol 8 to 10 mg/kg per hr until pneumonectomy was done; then propofol was used for all animals. Blood and liver samples were taken in four different moments: prepneumonectomy, prereperfusion, 10 min postreperfusion and 30 min postreperfusion to measure levels of interleukin (IL)-1β, IL-10, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, nuclear factor (NF)-κB, C-reactive protein, ferritin and caspase 3. Non-parametric test was used to find statistical meaning.
Results. Lung IR markedly increased the expression of TNF-α, IL-1β, MCP-1, NF-κB and caspase activity in control livers compared with basal levels, whereas liver IL-10 expression decreased 10 and 30 min post-reperfusion. Sevoflurane significantly decreased TNF-α, IL-1β, MCP-1, NF-κB liver expression and caspase 3 activity. Sevoflurane also reverted the lung IR-induced decrease in IL-10 expression.
Conclusions. The present results indicate that lung IR caused hepatic injury. Sevoflurane attenuated liver injury in a model of upper lobe left lung autotransplant in pigs.

Keywords: Liver ischemia-reperfusion injury, Lung transplantation, Sevoflurane.

(*Transplantation* 2014;98: 1151–1157)

One of the problems related to transplant surgery is the ischemia-reperfusion injury (IRI). Ischemia-reperfusion injury can damage not only the transplanted organ, but also distant ones (*1, 2*). Lung transplant surgery is a complex procedure and previous studies have observed that lung IRI consequent to lung transplant induces local and systemic inflammatory response with damage to remote organs (*3*). Liver seems to be particularly sensitive to IRI both when it is directly (*4, 5*) and indirectly (*1, 2, 6, 7*) involved by the transplant surgery. Different methods have been developed to mitigate IRI with relative success. One of them is anesthetic preconditioning (APC) with volatile anesthetics (sevoflurane, halothane, and isoflurane, among others). Volatile anesthetics exhibit anti-inflammatory effects in different organs and systems (*8–11*). In the case of the lung, APC with isoflurane and sevoflurane has shown its ability to attenuate IRI in isolated rat and rabbit lungs (*12, 13*) and to decrease the inflammatory response and the oxidative stress in a live ischemia-reperfusion

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(IR) lung model (*14*). After major thoracic surgical procedures it is not frequent to observe a hepatic injury as a clinical phenomenon. However, it can be assumed that there might be subclinical alterations if the liver responds to IRI in a remote organ. To our knowledge, these possible alterations have not been previously investigated. Therefore, the present study was designed to investigate the liver inflammatory response secondary to lung IRI in an in vivo upper lobe left lung autotransplant model and to evaluate a possible protective effect of sevoflurane.

RESULTS

There were no differences among SHAM, CON, and SEV groups in terms of animal weight (32±2.5; 42±3.8 and 35.8±2.9 kg respectively) or duration of the entire procedure (291±9.61; 287.4±35.3 and 305±29.63 min respectively). There were no differences between CON and SEV groups in terms of lung ischemia time (102±18 vs. 109±12 min). Moreover, there were no differences between the CON and SEV groups in terms of one-lung ventilation time (169±71 vs. 180±87 min).

Hemodynamic and Blood Gas Analysis

The hemodynamic and arterial gasometric values showed high stability (Table 1).

Interleukin-1β

Lung IR markedly increased interleukin (IL)-1β messenger RNA (mRNA) expression at prereperfusion (PRp) (P=0.028), postreperfusion (Rp)-10′ (P=0.002) and Rp-30′ (P=0.002) in CON but not in SHAM livers (Fig. 1A). Interleukin-1β mRNA expression was increased at Rp-10′ and Rp-30′ in SEV group also (SEV Rp-10′ vs. SHAM RP-10′, P=0,008; SEV Rp-30′ vs. SHAM Rp-30′, P=0.045) (Fig. 1A) but this rise was significantly lower than the one observed in CON group (CON Rp-10′ vs. SEV Rp-10′, P=0.05; CON Rp-30′ vs. SEV RP-30′, P=0.05) (Fig. 1A). Lung IR markedly increased the protein expression of IL-1β in CON group livers compared with CON prepneumonectomy (PPn) levels (P=0.00009) and with SHAM levels (CON Rp-10′ vs. SHAM Rp-10′, P=0.004; CON Rp-30′ vs. SHAM Rp-30′, P=0.010) (Fig. 1B). This rise was significantly higher (P<0.05) than the one observed in SEV group at Rp-10′ and Rp-30′ (Fig. 1B).

Tumor Necrosis Factor-α

Tumor necrosis factor (TNF)-α mRNA expression in liver tissue increased at PRp in the CON group but not in the SHAM or in the SEV groups (CON PRp vs. SHAM PRp P=0.05; CON PRp vs. SEV PRp P=0.003) (Fig. 1C). Lung IR markedly increased the protein expression of TNF-α in CON group livers compared to their PPn levels (P=0.00009) and with SHAM levels (CON vs. SHAM at PRp, Rp-10′ and RP-30′; P=0.002, P=0.009, and P=0.028 respectively) (Fig. 1D). This rise was significantly higher than the one observed in SEV group at PRp (P=0.003), Rp-10′ (P=0.014) and Rp-30′ (P=0.003) (Fig. 1D).

Interleukin-10

After lung reperfusion a decrease of IL-10 mRNA expression was observed in the liver of CON animals but not in SEV or SHAM livers (CON Rp-10′ vs. SEV Rp-10′, P=0.014; CON Rp-30′ vs. SHAM RP-30′, P=0.0006) (Fig. 1E). No significant differences for the protein expression of IL-10 were observed (Fig. 1F).

Monocyte Chemotactic Protein-1

Lung IR markedly increased the expression of mRNA monocyte chemotactic protein (MCP)-1 in liver tissue of CON

TABLE 1. Hemodynamic and gasometric data

	Mean blood pressure	Cardiac frequency	Cardiac index	pH	PO_2
SHAM					
Base	96.8±8.1	102.8±6.49	5.76±0.65	7.57±0.03	340±64.86
PPn	92.6±7.13	105.4±4.95	5.89±0.45	7.51±0.04	408.95±43.75
PRp	98.8±8.03	114.2±12.3	6.02±0.59	7.45±0.07	417±43.85
Rp-10′	94.2±5.64	110.6±13.73	6.46±0.34	7.5±0.04	450.6±32.2
Rp-30′	97±6.26	110.4±12.96	5.94±0.49	7.52±0.03	428.2±34.11
CONTROL					
Base	99±4.24	97.67±7.08	5.67±0.39	7.53±0.03	360.78±51.21
PPn	101.3±4.23	91.6±8.45	5.35±0.55	7.45±0.03	222.44±37.85
PRp	90.2±4.11	100.6±9.36	4.439±0.4	7.45±0.03	327.67±45.3
Rp-10′	96.2±4.92	93.4±7.55	5±0.52	7.44±0.03	270.44±47.72
Rp-30′	82.78±5.52	92.8±8.55	5.84±1.18	7.44±0.04	322.56±46.45
SEV					
Base	76.2±4.3	107.88±7.9	7.81±0.97	7.55±0.02	291.11±38.21
PPn	83.3±6.78	105±5.53	5.35±0.55	7.49±0.02	163±24.56
PRp	80.89±4.37	96.6±8.39	5.58±0.66	7.47±0.03	327.44±44.72
Rp-10′	69.4±4.69	101.6±6.97	6±1.16	7.43±0.03	330.67±48.7
Rp-30′	71.89±5.46	114.6±8.85	5.94±0.49	7.44±0.04	341.33±53.68

Data are expressed as the mean±standard error of the mean.

SHAM, sham-operated group; CON, control group; SEV, sevoflurane group; Base, basal; PPn, prepneumonectomy; PRp, prereperfusion; Rp-10′, 10 min postreperfusion; Rp-30′, 30 min postreperfusion; PO_2, partial pressure of oxygen.

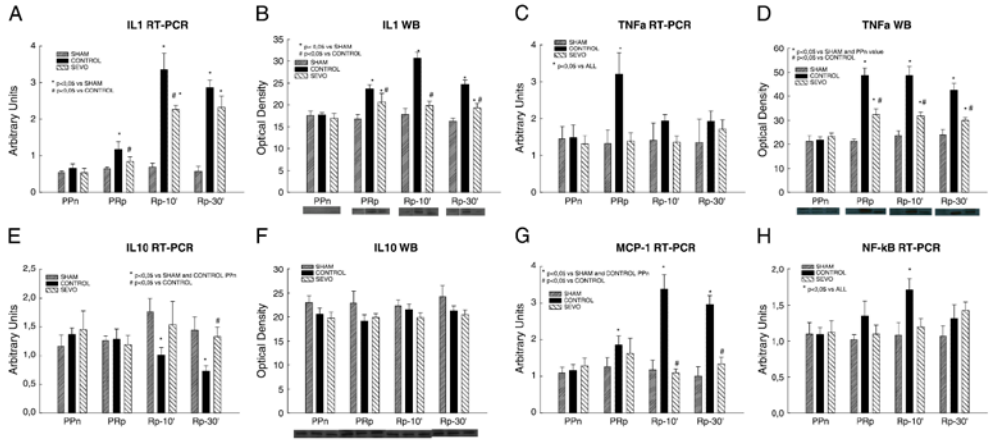

FIGURE 1. Bar graphs show the expression of proinflammatory and anti-inflammatory mediators in liver tissue throughout the experiment and compare the control group (CON) with the Sevoflurane group (SEV) and the Sham group (SHAM) results. A, IL-1β mRNA expression; (B) IL-β protein expression; (C) TNF-α mRNA expression; (D) TNF-α protein expression; (E) IL-10 mRNA expression; (F) IL10 protein expression; (G) MCP-1 mRNA expression; (H) NFκB mRNA expression. IL-1β, interleukin-1 beta; TNF-α, tumour necrosis factor-α MCP-1, monocyte chemoattractant protein-1; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells. PPn,Prepneumonectomy; PRp, Prereperfusion; Rp-10′, 10 min postreperfusion; Rp-30′, 30 min postreperfusion.

at PRp (P=0.05), Rp-10′ (P=0.00006) and Rp-30′ (P=0.0006) compared with PPn levels. These increases were not observed in SEV and SHAM groups (Fig. 1G).

Nuclear Factor-κB

Lung IR markedly increased the expression of nuclear factor (NF)-κB mRNA in liver tissue of CON group at Rp-10′ (P=0.003). This rise was not observed in SHAM and SEV groups (Fig. 1H).

Caspase 3

Lung IR markedly increased the caspase 3 in liver tissue of CON group at PRp (P=0.013), Rp-10′ (P=0.013), and Rp-30′ (P=0.013) compared with PPn levels. This increase was not observed in SHAM group and only at PRp in SEV group (P=0.013) (Fig. 2).

Blood Inflammatory Markers

No significant changes were observed in blood inflammatory markers of SHAM group throughout the experiment (Table 2).

Tumor Necrosis Factor-α

Lung IR markedly increased the blood levels of TNF-α of CON group at PRp (P=0.000001), Rp-10′ (P=0.0002), and Rp-30′ (P=0.0015) compared with PPn levels. Increased levels of TNF-α were also observed in the SEV group 30 min after reperfusion (PPn vs. Rp-30′, P=0.019), but this increase was significantly lower than the one observed in CON group at Rp-10′ (CON vs. SEV, P=0.000001) and Rp-30′ (CON vs. SEV, P=0.004).

Interleukin-1β

Lung IR markedly increased the blood levels of IL-1β of CON group at PRp (P=0.00008), Rp-10′ (P=0.000001), and Rp-30′ (P=0.00002) compared with PPn levels. Increased levels of IL-1β were also observed in the SEV group before reperfusion (PPn vs. PRp, P=0.004) and after it (PPn vs. Rp-10′, P=0.000002; PPn vs. Rp 30′, P=0.000001). However, after reperfusion, IL-1β plasma levels of SEV group were significantly lower than those of CON group (Rp-10′, P=0.00007; Rp-30′, P=0.0006).

C-reactive Protein

Lung IR increased the blood levels of C-reactive protein of CON group after reperfusion (Rp-10′, P=0.05; Rp-30′, P=0.02). This increase was significantly higher than that observed in the SEV group at Rp-10′ (P=0.045) and Rp-30′ (P=0.05).

Ferritin

No significant differences were observed in the plasma levels of ferritin.

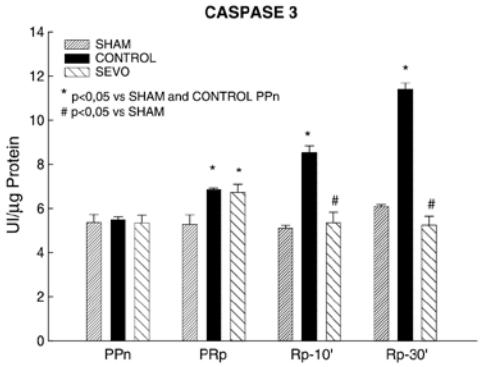

FIGURE 2. Bar graphs show the protein levels of Caspase 3 in liver samples of Control (CON), Sevoflurane (SEV) and Sham (SHAM) groups. PPn, Prepneumonectomy; PRp, Prereperfusion; Rp-10′, 10 min postreperfusion; Rp-30′, 30 min postreperfusion.

TABLE 2. Blood inflammatory markers

	PPn	PRp	Rp-10′	Rp-30′
TNF-α, pg/mL				
SHAM	54.093±4.44	54.405±3.3[a]	53.827±2.6[a]	53.213±2.46[a]
CON	52.735±1.58	155.04±2.55[b]	113.167±3.78[b,c]	83.123±2.86[b,c]
SEV	50.288±1.76	59.276±1.92[a]	54.938±0.84[a]	66.555±2.04[a,b]
IL-1β, pg/mL				
SHAM	8.362±0.33	8.532±0.27[a]	9.013±0.32[a,d]	8.763±0.29[a,d]
CON	8.52±0.28	15.673±0.25[b]	43.683±1.03[b,c]	45.649±1.17[b,c]
SEV	8.559±0.14	14.289±0.42[b]	31.903±1.14[a,b,c]	33.306±0.49[a,b,c]
CRP, ng/mL				
SHAM	0.115±0.016	0.092±0.019	0.090±0.018[a]	0.090±0.014[a]
CON	0.090±0.007	0.078±0.006	0.332±0.77[b,c]	0.151±0.014[b,c]
SEV	0.072±0.012	0.074±0.013	0.100±0.016[a]	0.097±0.013[a]
Ferritin, ng/mL				
SHAM	35.041±2.16	36.023±5.85	37.696±8.04	33.602±5.31
CON	31.558±3.48	28.316±3.47	31.418±4.26	37.263±6.22
SEV	42.351±11.32	37.94±11.74	36.222±9.24	47.345±12.22

[a] $P<0.05$ vs. CON group.
[b] $P<0.05$ vs. PPn.
[c] $P<0.05$ vs. PRp.
[d] $P<0.05$ vs. SEV group.
Data are expressed as the mean±standard error of the mean.
SHAM, sham-operated group; CON, control group; SEV, sevoflurane group; PPn, prepneumonectomy; PRp, prereperfusion; Rp-10′, 10 min postreperfusion; Rp-30′, 30 min postreperfusion; TNF-α, tumor necrosis factor alpha; IL-1ß, interleukin 1 beta, CRP; C-reactive protein.

Liver Enzyme Tests

Plasma levels of alanine aminotransferase, total bilirubin, conjugate bilirubin, total proteins, albumin, total globulins, and albumin-to-globulin ratio were within the normal range, and no difference was observed between groups.

Alkaline Phosphatase

Lung IR increased the plasma levels of alkaline phosphatase (AP) in the CON group at Rp-30′ compared to the SHAM (498±5 U/L vs. 286.5±44.5 U/L, $P=0.002$) and to the SEV (125±14 U/L, $P=0.002$) groups.

Aspartate Transaminase

Lung IR increased plasma levels of aspartate transaminase (AST) in the CON group at Rp-30′ compared to the SHAM (122.5±0.5 U/L vs. 83.5±7.5 U/L, $P=0.03$) group.

Gamma-Glutamyl Transpeptidase

Lung IR increased the plasma levels of gamma-glutamyl transpeptidase (GGT) in the CON group at Rp-10′ compared to the SHAM and SEV groups (42.5±1.5 vs. 24±5 and 16.5±5.5, respectively, $P=0.05$). This rise was even higher in the CON group at Rp-30′ compared to the SHAM and SEV groups (56±9 vs. 21±5 and 19.5±8.5, respectively, $P=0.046$).

DISCUSSION

Endothelial and epithelial dysfunctions often lead to IR lung injury during transplant surgery. This is characterized by postoperative nonspecific alveolar damage, lung edema, and hypoxemia. However, lung IRI can also induce a systemic inflammatory response that can subsequently damage remote organs. The liver seems to be particularly sensitive to circulating inflammatory mediators. Various studies have demonstrated that MCP-1 is the major chemoattractant for monocyte recruitment across endothelial cells as well as epithelial cells, both in vitro and in vivo (*15*, *16*). Monocyte chemotactic protein-1 plays an important role not only in recruiting cells of monocyte-macrophage lineage which contribute to the disease process (*17*) but also in modulating microcirculation (*18*). It has been observed that both TNF-α and Kupffer cells lead to an increase of MCP-1 mRNA transcription; however, TNF-α acts faster (<10 hr) than Kupffer cells' activation (24 hr) (*19*). In our study, it has been observed that lung IR markedly increased the expression of mRNA MCP-1 in liver tissue after reperfusion, once compared with PPn levels. Also, the protein expression of TNF-α was increased in the liver and blood samples of CON group after reperfusion. In a previous work from our group (*14*) that used the same animal model and surgical procedure, it was observed the presence of a local inflammatory injury with increased levels of oxidative and inflammatory mediators. In addition, TNF-α levels were increased in lung samples after reperfusion. Thus, it is likely that the increase in MCP-1 levels may be caused, at least in part, by the increased expression of TNF-α at both local and systemic levels. On the other hand, it has been observed that an imbalance between proinflammatory and anti-inflammatory cytokines seems to indicate greater damage. Interleukin-10 is regarded as the immunosuppressive cytokine for excellence; it acts on macrophages by suppressing the secretion of proinflammatory cytokines and increasing the production of cytokine inhibitors. It is

considered to be a protective cytokine because it can decrease the inflammatory response. In our study, it has been observed that IL-10 levels decreased in liver samples after reperfusion. At the same timepoints proinflammatory mediators were increased. This suggests the presence of an imbalance between proinflammatory and anti-inflammatory markers. The NF-κB is a complex protein that controls the transcription of DNA. The transcription factor NF-κB plays a key role in the regulation of inflammatory events associated with IR. Even though its role is still controversial (*20*), it has been associated with elevated mortality (*21, 22*). In a previous work by Jiang et al. (*23*) with a rat liver transplant model, increased expressions of TNF-α and IL-1β were observed in liver that migrated to the lung by liver outflow blood and activated NF-κB in lung inducing acute lung injury. In our study, it has been observed that NF-κB plays a role even in the opposite situation, that is, when the lung is directly damaged and it induces a hepatic injury. However, further investigation is required to use NF-κB as a possible prognostic marker.

All these results confirm, as previously observed (*3*), that lung IR is not just a local event but, through the entrance of inflammatory mediators into the systemic circulation, it affects the liver as evidenced by the increased levels of AP, AST, and GGT observed after reperfusion. Thus, because lung IR affects the liver, the treatment needs to focus on both local and remote responses. It has been previously proven that APC with volatile anesthetics exhibits a protective effect against IRI in different organs and systems (*8–14, 24–26*). Nevertheless, how this protective effect is provided remains unclear. There is evidence that volatile anesthetics can modulate the inflammatory response (*27*) and protect against oxidant injury (*28*). Regarding the inflammatory response, recent studies indicate that TNF-α plays an essential role in lung IR injury (*14, 29*), and it is capable of upregulating intercellular adhesion molecule 1 (*30*). The skill of volatile anesthetics to modulate the inflammatory response reducing post-ischemic adhesion of neutrophils (*31*), inhibiting neutrophil migration (*32*), and generating oxygen radicals by inflammatory cells (*33*) has already been proved. At the same time, the capacity to inhibit the release of TNF-α from cultured human peripheral mononuclear cells has been investigated (*34, 35*). However, to our knowledge, the effect of sevoflurane on remote tissue in case of lung IR has not been investigated. In a previous work from our group, it was observed that the administration of sevoflurane was able to reduce TNF-α and IL-1β levels in lungs after a lung transplantation surgery (*14*). In accordance with the previous work, the results from this study confirm the ability of sevoflurane APC to inhibit TNF-α and IL-1β release, even in remote organs. Likewise, in this study, it has been observed that sevoflurane APC was capable to reduce the mRNA expression of MCP-1 and NF-κB. Moreover, sevoflurane APC was able to avoid the decrease of IL-10 mRNA levels. This suggests that sevoflurane aids in preserving the balance between proinflammatory and anti-inflammatory cytokines. Interestingly, in this study, it has been observed that the mRNA and the protein expressions of IL-10 and IL-1β, considered in the same group and at the same time-point, showed different patterns. It is possible to suppose that this could be because of the different pathways that transcription and translation, as well as their regulation, follow. As previously mentioned, IR leads to the activation of cell death programs, including apoptosis. Many pathways and signals, both extracellular and intracellular, lead to apoptosis. Extracellular signals may include nitric oxide or cytokines. Hypoxia and increased intracellular calcium concentrations are considered as intracellular apoptotic signals. Caspases play the central role in the transduction of these apoptotic signals. There are two types of caspases: initiator caspases, (caspase 8, 10, 9, and 2) and effector caspases, (caspase 3, 7, and 6). The activation of initiator caspases requires binding to specific oligomeric adaptor proteins. Effector caspases are then activated by these active initiator caspases through proteolytic cleavage. The active effector caspases then proteolytically degrade a host of intracellular proteins to carry out the cell death program. In this study, the concentration of the effector caspase 3 was markedly increased in liver tissue of CON compared with SHAM group levels. TNF-α is the major extrinsic mediator of apoptosis. Also, NF-κB may modulate apoptosis during IR through a mechanism involving hypoxia-dependent inhibition of oxygen sensors (*36*). In our study, TNF-α and NF-κB levels were increased in the CON group compared with SHAM. Our results suggest that liver apoptosis could be consequent to the inflammatory damage. Moreover, our results showed that, after reperfusion, in SEV group, the levels of caspase 3 were significantly lower than those of the CON group which suggests that sevoflurane APC is not only able to manage the inflammatory imbalance that occurs in liver when lung transplant is performed, but can also reduce liver apoptosis secondary to lung IR. The effect of sevoflurane on apoptosis has been previously studied with contrasting results (*37–40*). This may be explained by the presence of different concentrations of proinflammatory and anti-inflammatory factors. Therefore, when ischemia and inflammation predominate, as in the case of lung transplantation, it may result more evident the antiapoptotic effect of sevoflurane. However, the mechanism by which sevoflurane exerts protective antiapoptotic effects in the liver secondary to lung IRI is not well known.

The present study may present some limitations linked to the selected model. First, the model of upper lobe left lung autotransplant used in this experimental setup may not reflect a clinical situation because the transplanted lung in the recipient in general has not been previously in contact with a volatile anesthetic before pneumonectomy in the donor. However, because it is accepted for its clinical uses, administering sevoflurane to the donor before the beginning of the surgery can be also performed in a clinic scenario. Secondly, this study only focused on a short interval of reperfusion time with an initiated injury. The choice is justified knowing that the main inflammatory changes are evident since the early phase after surgery. However, the possibility that the maximum level of inflammation can be reached in a longer period of reperfusion is not to be excluded (6–8 hr). For this reason, it may be interesting to investigate longer intervals of reperfusion. This may allow assessing a possible protection at a timepoint with more pronounced IRI and therefore have an impact on the detection of proteins because their production lags after changes in mRNA expression. In addition, in this model, a fixed dose of sevoflurane and the same precondition time were used in all subjects. Hence, further investigation is required to provide more detailed information about the interval of protection and the dose responses of APC with sevoflurane. Finally, this autotransplant model does not provide all the immunologic

issues that may play a role in the development of local and remote damages in clinical scenarios.

In conclusion, our findings suggest that (1) lung transplantation and the lung IRI that it causes affect the liver; (2) hepatic inflammatory injury increases hepatic apoptosis; (3) sevoflurane APC could be useful to reduce both the hepatic inflammatory and apoptotic response to lung IR. To our knowledge, this is the first study demonstrating the use of APC with sevoflurane to decrease liver inflammatory response in an in vivo upper lobe left lung autotransplant model.

MATERIALS AND METHODS

This study was granted approval by the institution's Research and Animal Experimentation Committee, and all animals received humane care in compliance with the European Convention on Animal Care.

Animal model and study groups

Twenty-one large white pigs, weighing 35 to 45 kg, underwent an orthotopic left upper lobe lung autotransplantation with a subsequent 30-min graft reperfusion. Animals were grouped by random numbers (Microsoft Excel 2003) to receive lung autotransplantation without sevoflurane APC (CON group, n=8) or with sevoflurane APC procedure (SEV group, n=8). In addition, five animals were submitted to sham surgery (Sham group).

Anesthesia and Surgical Protocol

The anesthesia protocol and the surgical technique for this lung autotransplant model have previously been described in detail (*14*). They are also described within the supplemental digital material (see **SDC**, http://links.lww.com/TP/B52)

Measurement and Sampling Timepoints

Baseline (Base) hemodynamic and arterial blood gas measurements were performed 30 min after the thoracotomy, with the animal under two-lung ventilation, but not lung biopsies or blood samples. Hemodynamic arterial gas measurements, blood samples, and liver biopsies were collected at the following timepoints: PPn—before completing pneumonectomy and with the animal under one-lung ventilation; PRp—before reperfusion and ventilation of the reimplanted left caudal lobe; 10 min Rp-10′—10 min after the reperfusion of the reimplanted lobe; and 30 min Rp-30′—30 min after the reperfusion of the reimplanted lobe.

Hemodynamic Measurements

A femoral-artery catheter was used to record mean blood pressure. In addition, the cardiac output monitor (Edwards Lifesciences, Irvine, CA) and thermodilution techniques were used at the mentioned timepoints to record the cardiac index.

Biochemical Studies in Liver Tissue

Liver-tissue biopsies were performed for biochemical studies. Every liver sample was placed in a cryotube, flash-frozen in liquid nitrogen, and stored at −80°C until biochemical analysis.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

RNA was isolated from liver samples of swines using the method described by Chomczynski (41), using the TRI Reagent Kit (Molecular Research Center, Inc., Cincinnati, OH), following the manufacturer's protocol. The purity of the RNA was estimated with 1.5% agarose gel electrophoresis, and the RNA concentrations were determined with spectrophotometry (260 nm). Reverse transcription of 2 μg of RNA for cDNA synthesis was performed using the Reverse Transcription System (Promega, Madison, WI), and a pd(N)6 random hexamer. Reverse transcription-polymerase chain reaction was performed using an Applied Biosystems 7300 apparatus with the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 300 nM concentrations of specific primers (Table 3). Reverse transcription-polymerase chain reaction amplifications were performed as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec. For the normalization of cDNA loading in the PCR, the amplification of 18S ribosomal RNA for every sample was used. Relative changes in gene expression were calculated using the 2-ΔΔCT method (*42*).

TABLE 3. Primer sequences

	Primers	Sequence (5′–3′)
18S	Forward	GGTGCATGGCCGTTCTTA
	Reverse	TCGTTCGTTATCGGAATTAACC
TNF-α	Forward	ATGAGAAGTTCCCAAATGGC
	Reverse	CTCCACTTGGTGGTTTGCTA
IL-1β	Forward	TGTGATGAAAGACGGCACAC
	Reverse	CTTCTTCTTTGGGTATTGTTTGG
IL-10	Forward	ACTGCACCCACTTCCCAGT
	Reverse	TTGTCCAGCTGGTCCTTTGT
MCP-1	Forward	AGCATCCACGTGCTGTCTC
	Reverse	GATCATCTTGCCAGTGAATGAGT
NFκB1	Forward	CAGCTCTTCTCAAAGCAGCA
	Reverse	TCCAGGTCATAGAGAGGCTCA

NF-κB1, nuclear factor; MCP, monocyte chemotactic protein; IL, interleukin; TNF, tumor necrosis factor.

Caspase 3 Activity Determination

Caspase 3 activity was determined by using colorimetric assay kit (Sigma-Aldrich, St. Louis, MO). The assay for caspase 3 is based on spectrophotometric detection of the chromophore pnitroanilide (pNA) after cleavage from the labeled substrate LEHD-pNA. The pNA light emission can be quantified using a spectrophotometer at 400 nm.

Western Blotting Analysis

Western blots were used to measure the protein expression of TNF-α, IL-1β, and IL-10 as previously described (*43*). Four samples from each timepoint of each group were analyzed.

Blood Inflammatory Markers

Femoral venous blood samples were collected for biochemical determinations at the following timepoints: PPn, PRp, Rp10′, and Rp30′. Each blood sample was stored in sterile polypropylene tubes and centrifuged at 2,000 rpm for 5 min. The plasma was separated, and samples were frozen at −80°C until assayed. Proinflammatory cytokines (TNF-α and IL-β), C-reactive protein, and ferritin were measured in plasma samples with enzyme-linked immunosorbent assay kits (MyBiosource, San Diego, CA) following the manufacturer's instructions.

Liver Enzyme Tests

Plasma samples were sent to the LAV laboratory (Madrid, Spain) to measure total bilirubin, conjugate bilirubin, AP, AST, alanine aminotransferase, GGT, total proteins, albumin, total globulins, and albumin:globulin ratio.

Statistical analysis

The data are expressed as the mean and the standard error of the mean (SE). Nonparametric tests were used. Accordingly, a Mann-Whitney *U* test was applied to establish differences between the analyzed groups. In addition, a Wilcoxon test for paired data was used to study the evolution of the intragroup values. Statistical significance was considered at a *P* value of 0.05 or less. The SPSS version 14.0 statistical package was used in this study.

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ARTÍCULO III

Intravenous lidocaine decreases tumor necrosis factor alpha expression both locally and systemically in pigs undergoing lung resection surgery.

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Intravenous Lidocaine Decreases Tumor Necrosis Factor Alpha Expression Both Locally and Systemically in Pigs Undergoing Lung Resection Surgery

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BACKGROUND: Lung resection surgery is associated with an inflammatory reaction. The use of 1-lung ventilation (OLV) seems to increase the likelihood of this reaction. Different prophylactic and therapeutic measures have been investigated to prevent lung injury secondary to OLV. Lidocaine, a commonly used local anesthetic drug, has antiinflammatory activity. Our main goal in this study was to investigate the effect of IV lidocaine on tumor necrosis factor α (TNF-α) lung expression during lung resection surgery with OLV.

METHODS: Eighteen pigs underwent left caudal lobectomy. The animals were divided into 3 groups: control, lidocaine, and sham. All animals received general anesthesia. In addition, animals in the lidocaine group received a continuous IV infusion of lidocaine during surgery (1.5 mg/kg/h). Animals in the sham group only underwent thoracotomy. Samples of bronchoalveolar lavage (BAL) fluid and plasma were collected before initiation of OLV, at the end of OLV, at the end of surgery, and 24 hours after surgery. Lung biopsy specimens were collected from the left caudal lobe (baseline) before surgery and from the mediastinal lobe and the left cranial lobe 24 hours after surgery. Samples were flash-frozen and stored to measure levels of the following inflammatory markers: interleukin (IL) 1β, IL-2, IL-10, TNF-α, nuclear factor κB, monocyte chemoattractant protein-1, inducible nitric oxide synthase, and endothelial nitric oxide synthase. Markers of apoptosis (caspase 3, caspase 9, Bad, Bax, and Bcl-2) were also measured. In addition, levels of metalloproteinases and nitric oxide metabolites were determined in BAL fluid and in plasma samples. A nonparametric test was used to examine statistical significance.

RESULTS: OLV caused lung damage with increased TNF-α expression in BAL, plasma, and lung samples. Other inflammatory (IL-1β, nuclear factor κB, monocyte chemoattractant protein-1) and apoptosis (caspase 3, caspase 9, and BAX) markers were also increased. With the use of IV lidocaine there was a significant decrease in the levels of TNF-α in the same samples compared with the control group. Lidocaine administration also reduced the inflammatory and apoptotic changes observed in the control group. Hemodynamic values, blood gas values, and airway pressure were similar in all groups.

CONCLUSIONS: Our results suggest that lidocaine can prevent OLV-induced lung injury through reduced expression of proinflammatory cytokines and lung apoptosis. Administration of lidocaine may help to prevent lung injury during lung surgery with OLV. (Anesth Analg 2014;119:815–28)

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Drs. Garutti and Rancan contributed equally to this work.

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The increased expression of cytokines observed after lung resection surgery (LRS) has been associated with the presence of postoperative infection and systemic inflammatory response syndrome, which worsen the postoperative course after thoracic surgery.[1,2] Up to 35% of patients undergoing LRS by thoracotomy present with symptoms of postoperative systemic inflammatory response syndrome.[2] The use of one-lung ventilation (OLV), which is necessary for administration of anesthesia in patients undergoing thoracic surgery, is associated with increased expression of cytokines in the lungs. This is probably one of the main reasons why an inflammatory reaction is more frequent in thoracic surgery (esophageal or pulmonary) using OLV than in intra-abdominal surgery.[3] Several pathophysiological mechanisms have been described to explain the lung injury that occasionally occurs during OLV. It seems that inflammation, generation of oxidative stress, barrier disruption (endothelial and epithelial), cell injury, and apoptosis

are involved in this injury.[4] Lidocaine is the oldest local anesthetic used in clinical practice and the only one that is approved for IV administration. In addition to blocking the inhibitory effects of the nerve signal, local anesthetics have systemic antiinflammatory properties,[5] which have proved beneficial in different types of surgeries[6] but have not been studied in procedures involving OLV. Various mechanisms of action have been proposed to explain the antiinflammatory effects of lidocaine. On one hand, it inhibits priming and migration of polymorphonuclear cells toward the inflamed area by diminishing their mobility and adhesion, with the resulting attenuation of the expression of cytokines and free oxygen radicals.[5,7,8] Inflammatory mediators are able to regulate a family of endopeptidases that break a variety of substrates of the extracellular matrix: the metalloproteinases (MMP). These proteinases have an important role in the tissue injury seen in neutrophil-dependent models of acute lung injury (ALI).[9] Lidocaine also acts on epithelial and endothelial lung cells and attenuates damage to these cells by cytokines.[10,11] The behavior of nitric oxide (NO) is a major determinant in protection against endothelial injury caused by oxidative stress or acute inflammation. In addition to inflammation, apoptosis plays an important role in the pathogenesis of ALI.[12] Increased apoptosis of pulmonary epithelial cells has been associated with the lung injury induced by mechanical ventilation (MV) and with the onset of lung damage in adult respiratory distress syndrome.[13] Therefore, we hypothesized that the use of lidocaine during LRS could moderate the intensity of inflammation and apoptosis within the lungs and that lidocaine could be useful to protect against the postoperative lung injury associated with LRS. The objectives of our study were to investigate ALI related to LRS and to assess the usefulness of IV lidocaine for attenuating both inflammation and apoptosis in pigs undergoing LRS. Our specific aims were to investigate the presence of local signs of increased inflammation (mainly tumor necrosis factor α [TNF-α] in bronchoalveolar lavage [BAL] samples) and apoptosis; to investigate possible systemic inflammatory alterations; and to investigate the effect of IV lidocaine administration on these alterations.

METHODS

This study was approved by the Research and Animal Experimentation Committee of the Complutense University of Madrid. All experiments were performed according to both European and Spanish laws for the handling and care of experimental animals. Special attention was paid to anesthesia and pain relief during surgical procedures.

Animal Model and Study Groups

Eighteen pigs with an average weight of 36 ± 10 kg underwent left thoracotomy for caudal lobectomy. Using Excel for PC (Microsoft Corp, Seattle, WA), they were randomly assigned to 3 groups (6 animals per group): lidocaine (LIDO) group, control (CON) group, and SHAM group. For animals in the LIDO group, lidocaine was administered as an initial bolus of 1.5 mg/kg followed by a continuous infusion of 1.5 mg/kg/h, which was maintained until the end of the procedure, whereas animals in the CON group received the same volume of 0.9% saline solution. The content of each syringe and infusion was administered blind. Animals from the SHAM group underwent left thoracotomy but not OLV or caudal lobectomy.

Anesthesia

The anesthetic protocol was the same for all animals. Drinking water was allowed ad libitum, but solid food was withheld for 18 hours before each experiment. Premedication was with intramuscular ketamine (10 mg/kg; Ketolar, Parke Davis, Pfizer, Dublin, Ireland). The animals were placed in the supine position and monitored using pulse oximetry and electrocardiography. Anesthetic induction was performed with propofol (3 mg/kg; Diprivan, AstraZeneca, Macclesfield, Cheshire, UK), fentanyl (3 μg/kg; Fentanest, Kern Pharmaceuticals, Houston, TX), and atracurium (0.6 mg/kg; Tracrium, GlaxoSmithKline, Brentford, UK). Maintenance was performed with a propofol infusion (10 mg/kg/h). Atracurium and fentanyl boluses were used when required. Fluid therapy was administered with lactated Ringer's solution at 5 mL/kg/h. Orotracheal intubation was performed with a 6- to 7-mm cuffed endotracheal tube placed 3 cm above the carina during a 2-lung ventilation (TLV) procedure. Throughout the process, volume-controlled MV was performed with lung-protective ventilation, a strategy based on a low tidal volume of 6 mL/kg, peak pressure <30 cm H_2O, positive end-expiratory pressure of 5 cm H_2O, and F_{IO_2} of 60%. The respiratory rate was set at 12 to 15 breaths per minute and the inspiration-to-expiration ratio at 1:2. Respiratory mechanics including peak inspiratory pressure, mean inspiratory pressure, and respiratory system compliance were constantly monitored throughout the experiments. Animals in the LIDO and CON groups underwent OLV. The procedure consisted of introducing the endotracheal tube into the right bronchus using a fiberoptic bronchoscope after the pleura was opened. This approach obviates ventilation of the tracheal bronchus. The surgeon ensured that the tube was correctly placed. OLV was maintained throughout the experiment (120 minutes); the tube was then moved back into the trachea, the position checked using a fiberoptic bronchoscope, and TLV was implemented. During OLV, the ventilator settings were maintained as during TLV (tidal volume of 6 mL/kg, peak pressure <30 cm H_2O, positive end-expiratory pressure of 5 cm H_2O, and F_{IO_2} of 60%). All animals were euthanized when the study was completed.

Surgical Protocol

After induction, the animals were placed in the right lateral decubitus position. Left thoracotomy was performed between the fifth and sixth ribs. Animals from the LIDO and CON groups underwent left caudal lobectomy lasting <120 minutes. Once the endotracheal tube was inserted into the right bronchus and OLV started, the procedure was performed. The dissection was accomplished through the interlobar fissure; the vein, artery, and caudal bronchus were individually ligated and sectioned. TLV was restored and re-expansion of the left cranial lobe verified. Next, a chest tube connected to a univalvular system (Heimlich) was inserted into the thorax, and the incision was closed. The propofol infusion was stopped when the surgeons

began to close the thoracic cavity to awaken the animal. Postoperative care included analgesia with ketorolac and/or dexketoprofen, antimicrobial prophylaxis with penicillin/benzathine, and water ad libitum. Animals from the SHAM group underwent the same protocol but without OLV or lung resection. After surgery, the animals were placed in a comfortable cage with water ad libitum, until the second procedure. Most animals were able to stand in their cages the following morning. Twenty-four hours after thoracotomy, general anesthesia was again administered using the protocol described above. Left thoracotomy was then performed to take a biopsy specimen from the lungs.

Hemodynamic Parameters and Arterial Gasometry

After anesthetic induction, the femoral vein was catheterized (inguinal cut-down) with a 7F triple-lumen catheter (Arrow-Howes) and the femoral artery with a PiCCO thermodilution catheter (PV2014L16 femoral artery in small adults Ø 4F, length 16 cm). The main hemodynamic variables, fluid responsiveness, preload, afterload, contractility, and pulmonary edema were measured using a PiCCO-Pulsion thermodilution monitor. Blood gas analyses were performed at baseline, 30 minutes after the start of OLV (30′ OLV), 120 minutes after the start of OLV (120′ OLV), 60 minutes after return to TLV (60′ TLV), and 24 hours after lobectomy (24 hours).

Bronchoalveolar Lavage Samples

BAL samples were taken at baseline, 120 minutes after the start of OLV (120′ OLV), 60 minutes after return to TLV (60′ TLV), and 24 hours after lobectomy (24 hours). Each lavage procedure involved squirting 20 mL of sterile saline into the distal bronchial division of the right lung and collecting it (after discharging the first 7 mL). The entire procedure was performed using a fiberoptic bronchoscope. Lavage fluid specimens recovered from each wash were centrifuged at 2500 rpm for 15 minutes and then frozen in liquid nitrogen and stored at −20°C until assayed.

Blood Samples

Femoral venous blood samples were taken for biochemical determinations. Blood samples were collected at baseline, 120 minutes after the start of OLV (120′ OLV), 60 minutes after return to TLV (60′ TLV), and 24 hours after lobectomy (24 hours). Each blood sample was stored in sterile polypropylene tubes and centrifuged at 2000 rpm for 5 minutes. The plasma was separated, and samples were frozen at −80°C until assayed.

Lung Samples

The left caudal lobe was biopsied immediately after the chest cavity was opened (base). The day after the intervention, biopsies were performed from the mediastinal lobe (ML) (not collapsed during OLV) and from the left cranial lobe (LCL) (collapsed during OLV). Each lung sample was placed into a cryotube immediately after extraction, flash-frozen in liquid nitrogen, and stored at −80°C until the biochemical determinations were made.

Wet-to-Dry Ratio

To quantify the wet-to-dry ratio, approximately 50 mg of each lung sample was incubated for 12 hours at 60°C and weighed again. The values obtained were analyzed with the following formula: [wet weight − dry weight]/wet weight.

Enzyme-Linked Immunosorbent Assays

Proinflammatory cytokines (interleukin [IL] 2 and TNF-α), antiinflammatory cytokines (IL-10), and MMP-2 and MMP-9 were measured in BAL and in plasma samples using specific commercial enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Bio-NOVA Científica Ltd, Madrid, Spain).

Preparation of Lung Homogenates and Determination of Caspase 3 and Caspase 9

Frozen organ samples were weighed and transferred to 50-mL polypropylene tubes (Falcon; Becton Dickinson, Lincoln Park, NJ) containing lysis buffer (4°C) at a ratio of 10 mL buffer/1 g of wet tissue. The lysis buffer consisted of 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Company), 1 mg/mL pepstatin A (Sigma Chemical Company), aprotinin (Sigma Chemical Company), and leupeptin (Sigma Chemical Company) in 1× phosphate-buffered saline solution (pH 7.2) (Biofluids, Rockville, MD) containing 0.05% sodium azide (Sigma Chemical Company). The samples were homogenized for 30 seconds with an electrical homogenizer (Polytron; Brinkmann Instruments, Westminster, NY) at maximum speed, and the tubes were immediately frozen in liquid nitrogen. The samples were homogenized 3 times for optimal processing. The homogenates were later thawed in a 37°C water bath and centrifuged at 119,000*g* (1 hour, 4°C) to separate cellular organelles. The supernatants were frozen at −80°C to enable the formation of macromolecular aggregates. After thawing at 4°C, the aggregates were pelleted at 3000*g* (4°C), and the final homogenate volume was measured with a graduated pipette. The homogenates were stored at −80°C until they were assayed for the quantitative presence of caspases. An enzyme-linked immunosorbent assay kit was used according to the manufacturer's instructions (Sigma Chemical Company).

NOx Determinations

The concentration of nitric oxide (NOx) metabolites was measured in BAL and in blood samples using the Griess test, which determined the concentration of NO_2 after reduction of NO_3 to NO_2. Reproducibility within the assays was evaluated in 3 independent experiments, and each assay was performed with 3 replicates. The overall intra-assay coefficient of variation was calculated to be <5%. Assay-to-assay reproducibility was evaluated in 3 independent experiments. The overall interassay coefficient of variation was calculated to be <6%.

Western Blotting

Western blotting was used to measure levels of TNF-α, IL-1β, IL-10, Bad, Bax, Bcl-2, inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS). Briefly, after homogenization with lysis buffer, tissue

samples (50–60 mg) were boiled with gel-loading buffer (0.100 M Tris-Cl; 4% sodium dodecyl sulfate; 20% glycerol; 0.1% bromophenol blue) at a 1:1 ratio and sonicated. The protein concentrations were determined using the bicinchoninic acid assay. The total protein equivalents (25 μg) for each sample were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% acrylamide gels and transferred onto a nitrocellulose membrane using a semidry transfer system. The membrane was immediately placed in blocking buffer containing 5% nonfat milk in 20 mM Tris (pH 7.5), 150 mM NaCl, and 0.01% Tween-20. The blot was allowed to block at 37°C for 1 hour. The membrane was incubated with anti-rat rabbit polyclonal TNF-α, IL-1β, IL-10, Bad, Bax, Bcl-2, iNOS, and eNOS (dilution 1:1000) antibodies for 2 hours at 25°C to 27°C or for 12 hours at 4°C, followed by incubation with an anti-rabbit horseradish peroxidase–conjugated IgG antibody (1:2000). After washing with T-TBS, the membranes were incubated with ECL Plus detection reagents (Amersham Life Science Inc., Buckinghamshire, UK) and exposed to X-ray film. The films were scanned using a densitometer (BioRad GS 800) to determine the relative optical densities. Prestained protein markers were used for molecular weight determinations, and the bands were analyzed using Quantity ONE 1.0 Analysis Software, version 4.5.2.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction

RNA was isolated from lung samples according to the method described by Chomczynski and Sacchi[14] using the TRI Reagent Kit (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's protocol. The purity of the RNA was estimated using 1.5% agarose gel electrophoresis, and the RNA concentrations were determined using spectrophotometry. Reverse transcription of 2 mg of RNA for cDNA synthesis was performed using the Reverse Transcription System (Promega, Madison, WI, USA) and a pd(N)6 random hexamer. Reverse transcription polymerase chain reaction was performed using an Applied Biosystems 7300 apparatus with the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 300-nM concentrations of specific primers. The sequences of the primer transcripts were as follows: monocyte chemoattractant protein-1 (MCP-1), forward ACAGAAGAGTCACCAGCAGCAA, reverse GCCCGCGATGGTCTT; IL-1, forward TGTGATGAAA GACGGCACAC, reverse CTTCTTCTTTGGGTATTGTTTGG; TNF-α, forward ATGAGAAGTTCCCAAATGGC, reverse CTCCACTTGGTGGTTTGCTA; IL-10, forward ACTGCA CCCACTTCCCAGT, reverse TTGTCCAGCTGGTCCTTTGT; nuclear factor κB (NFκB), forward CAGCTCTTCTCAA AGCAGCA, reverse TCCAGGTCATAGAGAGGCTCA; and iNOS, forward CTTTGCCACGGACGAGAC, reverse TCATTGTACTCTGAGGGCTGAC. The housekeeping gene 18s was used as an internal control (forward GGTGC ATGGCCGTTCTTA, reverse TCGTTCGTTATCGGAATT AACC). Relative changes in mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method.[15]

Statistics

The 3 groups (SHAM, CON, and LIDO) were compared for plasma, BAL, and lung tissue. Each parameter was analyzed separately. In lung tissue, ML and LCL values were compared separately. The Kruskal-Wallis test was used to identify any significant difference among the groups (the null hypothesis was rejected with an α value = 0.05). Then the Mann-Whitney *U* test was used to analyze the specific sample pairs for significant differences. Statistical significance was set at $P \leq 0.003$. All data were expressed as mean ± standard deviation.

RESULTS

The main result of this work was the increase of TNF-α expression observed in BAL and plasma samples in the CON group as a direct consequence of LRS with OLV (120′ OLV) (Figs. 1A and 2A). In both plasma and BAL samples, it was observed that the administration of lidocaine was able to significantly reduce this increase (Figs. 1A and 2A). In addition, TNF-α expression was increased in CON group plasma samples even after reperfusion (60′ TLV and 24 hours), and once again the administration of lidocaine was able to significantly reduce this increase (Fig. 1A).

General and Cardiorespiratory Variables

No differences were observed among the SHAM, CON, and LIDO groups in terms of weight (46.70 ± 18.18 kg; 34.55 ± 10.82 kg; and 36.45 ± 8.47 kg, respectively) or duration of the procedure (179.76 ± 24.86 minutes; 180.34 ± 6.50 minutes; and 183.56 ± 31.78 minutes).

Hemodynamic values, blood gas values, and airway pressures remained very stable during the procedure and the day after surgery (Tables 1 and 2). The only significant difference observed between the SHAM group and the other 2 groups was caused by the different number of ventilated lungs so that SHAM group animals had higher blood oxygenation and lower peak airway pressures than the LIDO and CON groups (Table 2).

Plasma Variables

TNF-α levels increased in CON group plasma samples at 120′ OLV, 60′ TLV, and 24-hour time points compared with the SHAM group levels. In the LIDO group, the levels of TNF-α only increased at the 24-hour time point compared with the SHAM group levels (Fig. 1A). Additional changes were observed in the inflammatory markers studied in the plasma samples: at 60′ TLV time point, the IL-2 values were higher in the CON group than in the LIDO group (Fig. 1B); increased levels of MMP-2 and MMP-9 were observed at the 24-hour time point in the CON group (Fig. 1, C and D). Plasma values of IL-10 were below the levels detectable in the laboratory.

BAL Biomarker Variables

TNF-α levels increased in BAL CON group samples at 120′ OLV, 60′ TLV, and 24-hour time points compared with SHAM group levels. In the LIDO group, the levels of TNF-α only increased at 120′ OLV and 60′ TLV time points compared with SHAM group levels. However, this increase was less than the one observed in the CON group at the 120′ OLV time point (Fig. 2A). Additional changes were observed in

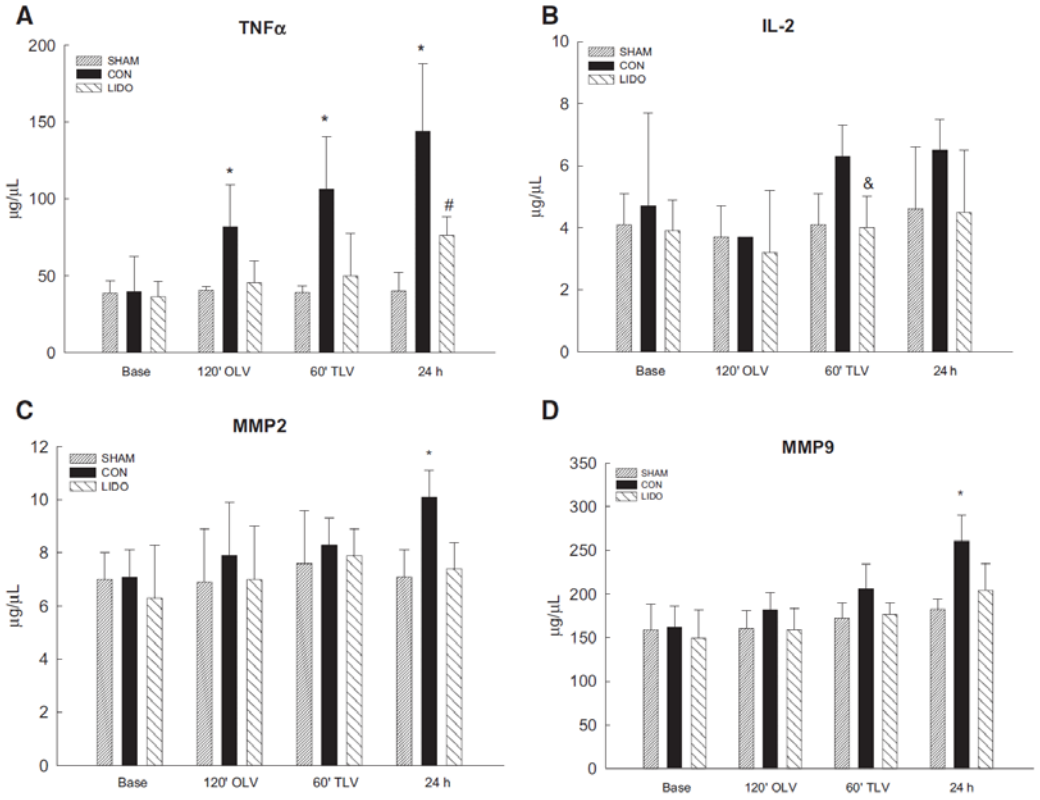

Figure 1. Plasma biomarkers. Bar graphs show the expression of proinflammatory cytokines and metalloproteinases in plasma samples throughout the experiment and compare the control group (CON) with the lidocaine group (LIDO) and with the SHAM group. (A), TNF-α; (B) IL-2; (C) MMP-2; (D) MMP-9. Base = Baseline; 120′ one-lung ventilation (OLV) = 120 minutes after the beginning of one-lung ventilation; 60′ two-lung ventilation (TLV) = 60 minutes after the beginning of 2-lung ventilation; 24 h = 24 h after surgery. *P < 0.003 CON group versus SHAM group; #P < 0.003 LIDO group versus SHAM group; &P < 0.003 LIDO versus CON group.

the inflammatory markers studied in the BAL samples: significantly increased levels of MMP-2 and MMP-9 were observed in the BAL CON group samples at 120′ OLV, 60′ TLV, and 24-hour time points (Fig. 2, D and E); a significant reduction of MMP-2 levels was observed at the 24-hour time point in the LIDO group (Fig. 2D).

Wet-to-Dry Ratio

In the CON group samples, lung edema was increased in both the ML and the LCL 24 hours after surgery. Lidocaine administration attenuated these changes even if the P values did not reach 0.003 (ML lobe P = 0.008 and LCL lobe P = 0.008) (Fig. 3).

Lung Biopsy Variables

In the lung biopsy samples collected at the 24-hour time point, an increase was observed in TNF-α mRNA expression in the LCL of the CON group compared with the levels of the SHAM and the LIDO groups (Fig. 4A). TNF-α protein expression of the ML and the LCL samples was increased in the CON group compared with the SHAM and the LIDO group levels (Fig. 4B). Additional changes of other inflammatory markers (IL-1β, NFκB, and MCP-1) were observed in the samples of lung biopsies collected from the CON group that affected both ML and LCL (Fig. 4). Lidocaine administration was able to attenuate these changes (Fig. 4). Moreover, in the CON group lung biopsies, we observed an increased expression of proapoptotic biomarkers (caspase 3, caspase 9, BAD, and BAX) and decreased expression of the antiapoptotic marker Bcl-2. These changes affected both ML and LCL and were attenuated by the administration of lidocaine (Fig. 5).

Nitric Oxide Metabolism

Levels of NOx were decreased in both the plasma and the BAL samples of the CON group during surgery and 24 hours later. No changes were observed in the SHAM group. LIDO group values were generally higher than CON group values (Fig. 6, A and B), suggesting that IV administration of lidocaine was also effective in reducing the alterations of NOx levels in BAL and plasma. mRNA expression of iNOS increased in the LCL in both the LIDO and the

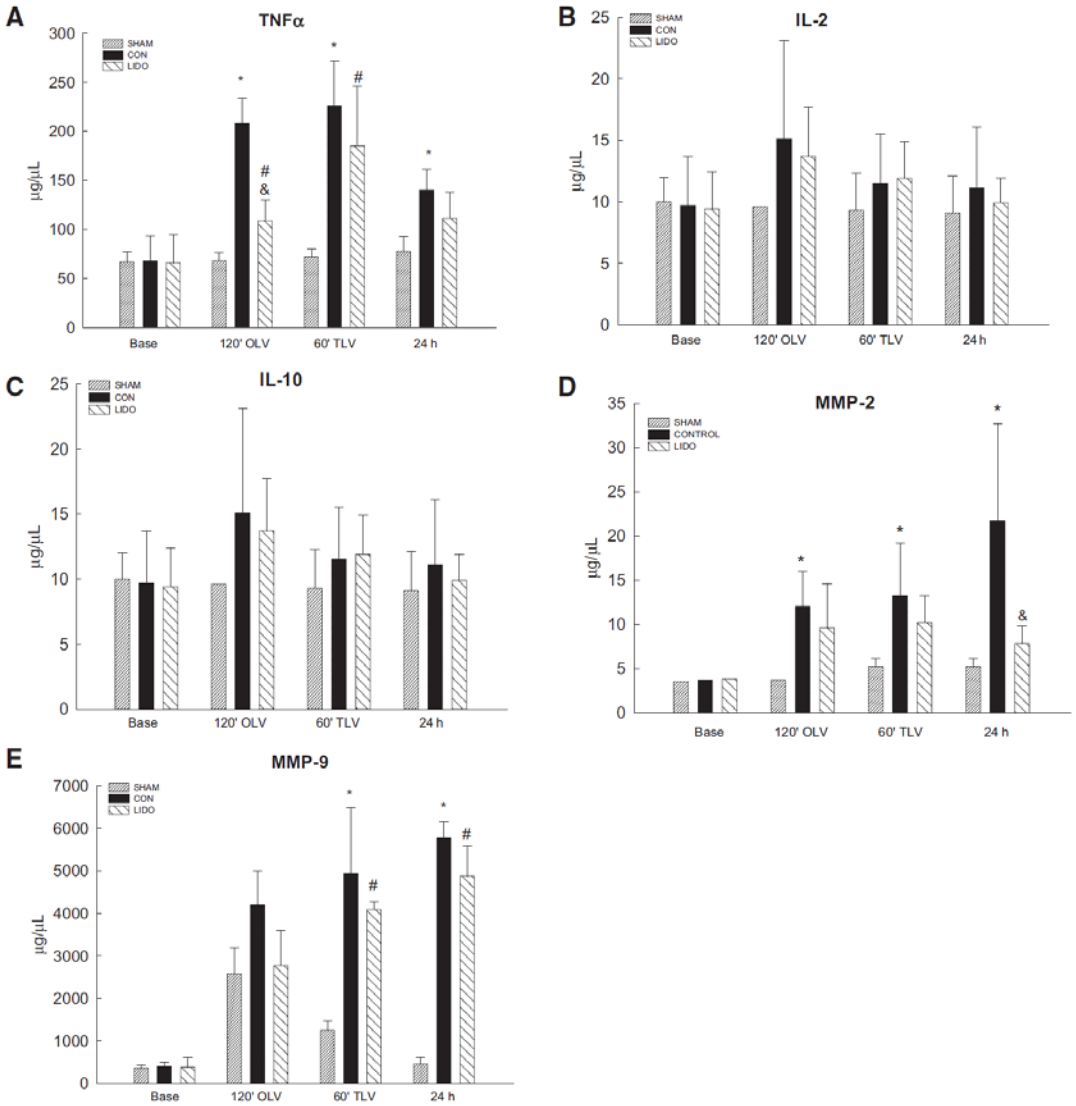

Figure 2. Bronchoalveolar lavage (BAL) markers. Bar graphs show the expression of inflammatory cytokines and metalloproteinases in BAL samples throughout the experiment and compare the control group (CON) with the lidocaine group (LIDO) and with the SHAM group. *P < 0.003 CON group versus SHAM group; #P < 0.003 LIDO group versus SHAM group; &P < 0.003 LIDO group versus CON group. (A), TNF-α; (B) IL-2; (C) IL-10; (D) MMP-2; (E) MMP-9. TNF = tumor necrosis factor; IL = interleukin; MMP = metalloproteinases; base = baseline; 120′ one-lung ventilation (OLV) = 120 minutes after the beginning of one-lung ventilation; 60′ two-lung ventilation (TLV) = 60 minutes after the beginning of 2-lung ventilation; 24 h = 24 hours after surgery.

CON groups, with no significant differences between them (Fig. 6C). Protein expression of iNOS was increased in both lobes in the CON group. However, in this case, lidocaine administration was able to attenuate this increase in the ML (Fig. 6D). Protein expression of eNOS was significantly increased in the LCL of the LIDO group compared with the CON group (Fig. 6E).

DISCUSSION

Intraoperative use of IV lidocaine was able to reduce postoperative lung injury in our experimental model of LRS. These changes were evident in both lungs, and IV lidocaine was able to attenuate them. Also, a systemic inflammatory response was observed in this study, and the IV administration of lidocaine was able to decrease it. Different molecular mechanisms can explain the protection afforded by

Table 1. Hemodynamic Values

		Baseline	30′ OLV	120′ OLV	60′ TLV	24 h
HR (bpm)	CON	94.6 ± 14	101.4 ± 18	90.8 ± 8	96.8 ± 8	82.8 ± 16
	LIDO	85.2 ± 12	104.2 ± 12	107.8 ± 25	108.8 ± 9	94.8 ± 11
	SHAM	79.0 ± 24	77.5 ± 13	88.0 ± 17	87.8 ± 10	95.0 ± 16
MAP (mm Hg)	CON	92 ± 27	100 ± 22	97 ± 10	124 ± 17	104 ± 10
	LIDO	99 ± 16	98 ± 22	102 ± 18	119 ± 10	98 ± 15
	SHAM	93 ± 11	94 ± 12	102 ± 11	114 ± 14	106 ± 22
CVP (mm Hg)	CON	8.2 ± 5.5	9.6 ± 4.3	9.2 ± 3.8	8.4 ± 4.5	8.8 ± 4.8
	LIDO	7.8 ± 5.8	7.4 ± 6.6	7.8 ± 4.8	6.2 ± 6.9	7.4 ± 5.3
	SHAM	9.7 ± 3.8	9.3 ± 5.2	10.5 ± 4.8	9.2 ± 5.5	6.7 ± 3.4
CI (L/min/m^2)	CON	3.47 ± 0.8	3.42 ± 0.7	3.14 ± 0.4	3.74 ± 1.0	2.84 ± 0.9
	LIDO	3.75 ± 1.2	3.77 ± 1.1	3.11 ± 0.9	3.90 ± 1.0	2.67 ± 0.4
	SHAM	2.73 ± 0.6	2.79 ± 0.7	2.83 ± 1.2	3.45 ± 1.1	3.02 ± 1.1
GEDVI (L/min/m^2)	CON	546 ± 249	541 ± 258	514 ± 83	626 ± 147	395 ± 58
	LIDO	590 ± 154	605 ± 136	431 ± 156	483 ± 135	471 ± 183
	SHAM	497 ± 156	497 ± 156	519 ± 187	580 ± 136	429 ± 149
dpmax (mm Hg/s)	CON	636 ± 176	632 ± 230	722 ± 108	782 ± 244	746 ± 250
	LIDO	746 ± 190	704 ± 147	792 ± 261	850 ± 383	916 ± 343
	SHAM	765 ± 210	715 ± 211	705 ± 144	830 ± 220	814 ± 289
ELWI (mL)	CON	17.0 ± 10.4	14.0 ± 8.5	14.8 ± 5.4	18.6 ± 7.5	9.3 ± 4.2
	LIDO	17.6 ± 9.9	17.8 ± 9.5	13.2 ± 8.2	14.6 ± 10.4	8.2 ± 2.5
	SHAM	8.5 ± 2.7	8.5 ± 2.7	13.3 ± 5.5	14.2 ± 5.4	7.5 ± 1.2
SVV (%)	CON	18.4 ± 6.2	15.2 ± 7.9	15.0 ± 2.7	14.6 ± 4.3	17.8 ± 7.4
	LIDO	15.4 ± 4.9	15.6 ± 3.6	11.0 ± 4.6	16.6 ± 3.6	24.4 ± 5.3
	SHAM	19.0 ± 5.4	9.3 ± 1.8	11.2 ± 6.6	18.0 ± 5.1	25.7 ± 9.1
SVRI (dyn/s/cm^{-5}/m^2)	CON	1899 ± 496	2139 ± 655	2349 ± 405	2739 ± 664	2798 ± 1185
	LIDO	2270 ± 1089	2213 ± 1111	3008 ± 1600	2595 ± 884	2893 ± 629
	SHAM	2897 ± 939	2680 ± 733	3126 ± 1383	3067 ± 1604	2948 ± 1050

Data are expressed as mean ± standard error of the mean.

HR = heart rate; MAP = mean arterial blood pressure; CI = cardiac index; CVP = central venous pressure; GEDVI = global end-diastolic volume; ELWI = extravascular lung water; SVV = stroke volume variation; SVRI = systemic vascular resistance index; CON = control group; LIDO = lidocaine group; SHAM = sham group; OLV = one-lung ventilation; TLV = two-lung ventilation; 30′ OLV = 30 minutes after the beginning of OLV; 120′ OLV = 120 minutes after the beginning of OLV; 60′ TLV = 60 minutes after the beginning of TLV; 24 h = 24 hours after surgery.

Table 2. Blood Gases and Respiratory Variables

	Group		Baseline	30′ OLV	120′ OLV	60′ TLV	24 h
Sao_2 (%)	CON	5	100 ± 0.0	98.6 ± 2.1	99.2 ± 1.3	99.0 ± 1.7	99.8 ± 0.4
	LIDO	5	98.8 ± 2.2	99.2 ± 0.8	99.8 ± 0.4	99.8 ± 0.4	100 ± 0.0
	SHAM	6	99.5 ± 1.2	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Pao_2 (mm Hg)	CON	5	236 ± 55	136 ± 24	176 ± 17	235 ± 78	245 ± 89
	LIDO	5	266 ± 103	149 ± 46	200 ± 30	235 ± 52	267 ± 43
	SHAM	6	299 ± 77	244 ± 70	256 ± 45	281 ± 51	252 ± 54
$Paco_2$ (mm Hg)	CON	5	41.4 ± 9.9	41.2 ± 7.8*	43.4 ± 6.8*	48.0 ± 9.7	34.8 ± 6.9
	LIDO	5	41.0 ± 11.0	41.4 ± 8.8*	45.2 ± 6.6*	46.2 ± 7.9	40.0 ± 3.0
	SHAM	6	37.3 ± 4.2	37.0 ± 3.4	41.3 ± 4.0	45.0 ± 8.7	39.2 ± 6.6
pH	CON	5	7.5 ± 0.1	7.5 ± 0.1	7.5 ± 0.1	7.4 ± 0.1	7.5 ± 0.1
	LIDO	5	7.5 ± 0.1	7.5 ± 0.1	7.4 ± 0.1	7.4 ± 0.1	7.5 ± 0.0
	SHAM	6	7.5 ± 0.0	7.5 ± 0.0	7.5 ± 0.0	7.5 ± 0.1	7.5 ± 0.1
PpAW (cm H_2O)	CON	5	28.4 ± 3.8	34.4 ± 6.4	34.8 ± 7.1	32.2 ± 9.7	31.6 ± 6.9
	LIDO	5	27.9 ± 3.3	36.4 ± 7.7	35.6 ± 5.4	31.2 ± 7.2	32.8 ± 7.4
	SHAM	6	28.2 ± 3.1	30.7 ± 4.7	30.5 ± 8.8	32.8 ± 11	31.6 ± 8.5
Mean P (cm H_2O)	CON	5	7.0 ± 1.0	8.4 ± 2.3	7.6 ± 1.7	7.8 ± 1.3	7.6 ± 1.1
	LIDO	5	7.0 ± 3.0	8.2 ± 3.3	8.6 ± 0.5	9.4 ± 1.9	8.8 ± 1.0
	SHAM	6	7.0 ± 0.6	7.3 ± 2.2	7.2 ± 1.7	7.7 ± 1.6	8.3 ± 1.8

Data are expressed as mean ± standard error of the mean.

Sao_2 = oxygen saturation; PpAW = pulmonary artery wedge pressure; mean P = mean pressure; CON = control group; LIDO = lidocaine group; SHAM = sham group; OLV = one-lung ventilation; TLV = two-lung ventilation; 30′ OLV = 30 minutes after the beginning of OLV; 120′ OLV = 120 minutes after the beginning of OLV; 60′ TLV = 60 minutes after the beginning of TLV; 24 h = 24 hours after surgery.

*$P < 0.001$ versus SHAM group.

lidocaine during LRS with periods of OLV. Some authors have demonstrated how lidocaine attenuated ALI in experimental models,[16,17] although this effect had not been studied in a model investigating lung damage such as that caused by LRS with OLV. The results of our porcine model of LRS showed that intraoperative IV lidocaine attenuated the systemic inflammatory response and decreased the intensity of lung injury. This effect was demonstrated by a decreased inflammatory response, maintenance of NO metabolism, and an antiapoptotic response. Although the exact mechanism of these effects is not clear, it seems that lidocaine provided protection both through its inhibitory

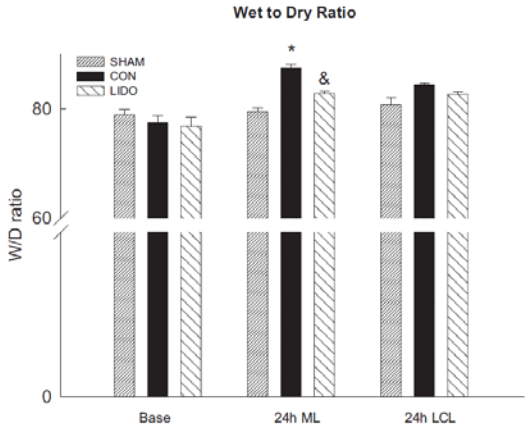

Figure 3. Wet-to-dry ratio. The bar graphs show the expression of the wet-to-dry ratio of lung biopsies comparing the control group (CON) with the lidocaine group (LIDO) and with the SHAM group. Base, lung biopsy of the left caudal lobe taken at the beginning of the surgical procedure; 24-h mediastinal lobe (ML), lung biopsy of the mediastinal lobe (not collapsed during surgery) taken 24 hours after surgery; 24-h left cranial lobe (LCL), lung biopsy of the left cranial lobe (collapsed during surgery) taken 24 hours after surgery. *P < 0.003 CON group versus SHAM group; &P < 0.003 LIDO group versus CON group.

effect on neutrophils[18] and through the cellular barriers that prevent extravasation of fluid from the pulmonary vascular system (vascular endothelial cells and epithelial cells).

Inflammation

It has been observed that thoracic surgery causes a greater inflammatory reaction than intra-abdominal surgery,[3] and some authors have related it to the use of OLV. In fact, it has been observed that OLV per se generates oxidative stress and phenomena of ischemia-reperfusion injury.[19,20] During OLV, ALI occurs, and this increases morbidity and mortality after LRS.[4] At the beginning of this process, alveolar macrophages increase their production of inflammatory mediators, in particular proinflammatory cytokines such as IL-1β and TNF-α or other mediators like NFκB.[21,22] In accordance with previous studies, our results show that LRS with OLV generates a significant local inflammatory response, which is mediated by IL-1β, TNF-α, NFκB, and MCP-1. Moreover, in this study, the presence of a systemic inflammatory response secondary to LRS has been confirmed, and our results suggest that TNF-α would play a pivotal role in the transmission of the inflammatory response from local to systemic. Another proinflammatory cytokine, IL-2, has been described in relation to lung injury. Yet, its role has not been defined, and it seems to be able to act positively[23] or negatively[24] depending on the situation. In this study, IL-2 levels, both local and systemic, did not seem to be deeply affected by surgery. This could suggest that this cytokine would act only in an initial stage of the inflammatory response without great systemic fallout. As previously observed,[25] the presence of lung edema was also observed in this study. Since the levels of MCP-1 were increased in both lungs, the edema could have been due, at least in part, to the presence of neutrophil infiltration. In this study, we observed that the IV administration of lidocaine was able to attenuate all inflammatory changes. It reduced the levels of proinflammatory mediators and increased the levels of antiinflammatory cytokines contributing to the balance conservation. Moreover, Hollmann et al.[26] showed that local anesthetics inhibit in a time-dependent and reversible manner G protein–coupled receptor signaling, which is involved in many processes relevant for perioperative medicine (e.g., coagulation and inflammation). Even if the model used in the study by Hollmann et al.[26] was different from ours, it could suggest that to administer lidocaine during the entire LRS surgery (180 minutes approximately in our model) would be enough to inhibit G protein–coupled receptor signaling with positive consequences on the inflammatory response.

Metalloproteinases

Inflammatory mediators such as cytokines and chemokines can regulate MMPs, which break a variety of substrates of the extracellular matrix. MMPs can modify the inflammatory reaction by modulating cytokines.[27] MMP levels increase in processes that trigger an inflammatory response,[28,29] including surgery and MV.[30,31] The 2 most widely studied MMPs in lung damage are MMP-9 and MMP-2. Secretion of MMP-9 by neutrophils favors transmembrane migration of neutrophils; inhibition of these MMPs is associated with reduced presence of neutrophils in the inflamed area.[32] MMP-2 is present in the vascular endothelium and alveolar epithelium and shares the substrate it acts upon (type IV collagen, gelatin) with MMP-9. The results of this study have shown an extraordinary hypersecretion of MMP-9 in BAL samples of animals that had undergone LRS with OLV during the first 24 hours after surgery (compared with animals that only underwent thoracotomy and TLV), and the MMP-9 expression in BAL specimens was 20-fold higher than blood samples in animals undergoing OLV. This finding reflects the important role of this MMP in lung injury during LRS. We speculate that the attenuation of hypersecretion of MMP-9 observed in our study is a response to the inhibitory effect of lidocaine on neutrophil activity. However, reduced expression of MMP-2 cannot be explained by this mechanism and is more likely associated with the effects of lidocaine on vascular endothelial cells and alveolar epithelial cells, which reduce adhesion of polymorphonuclear cells and hamper migration of neutrophils to the inflamed area.

Nitric Oxide Metabolism

Endothelial damage can cause a deficiency of NO production. The results of some studies indicate that lidocaine reduces the oxidative stress and lung damage caused by ischemia-reperfusion.[16,17] These findings have been explained by the inhibition of neutrophil adhesion and subsequent migration to the lung or by the antioxidant properties of lidocaine. Also, reactive nitrogen species are a key factor in inflammation, and alterations in NO synthesis during ischemia-reperfusion cause dysfunction of the pulmonary vasculature.[33] Excessive NO production of iNOS may have an important role in tissue injury secondary to an inflammatory response.[34,35] Overproduction of iNOS decreases the amount of available NO, and a

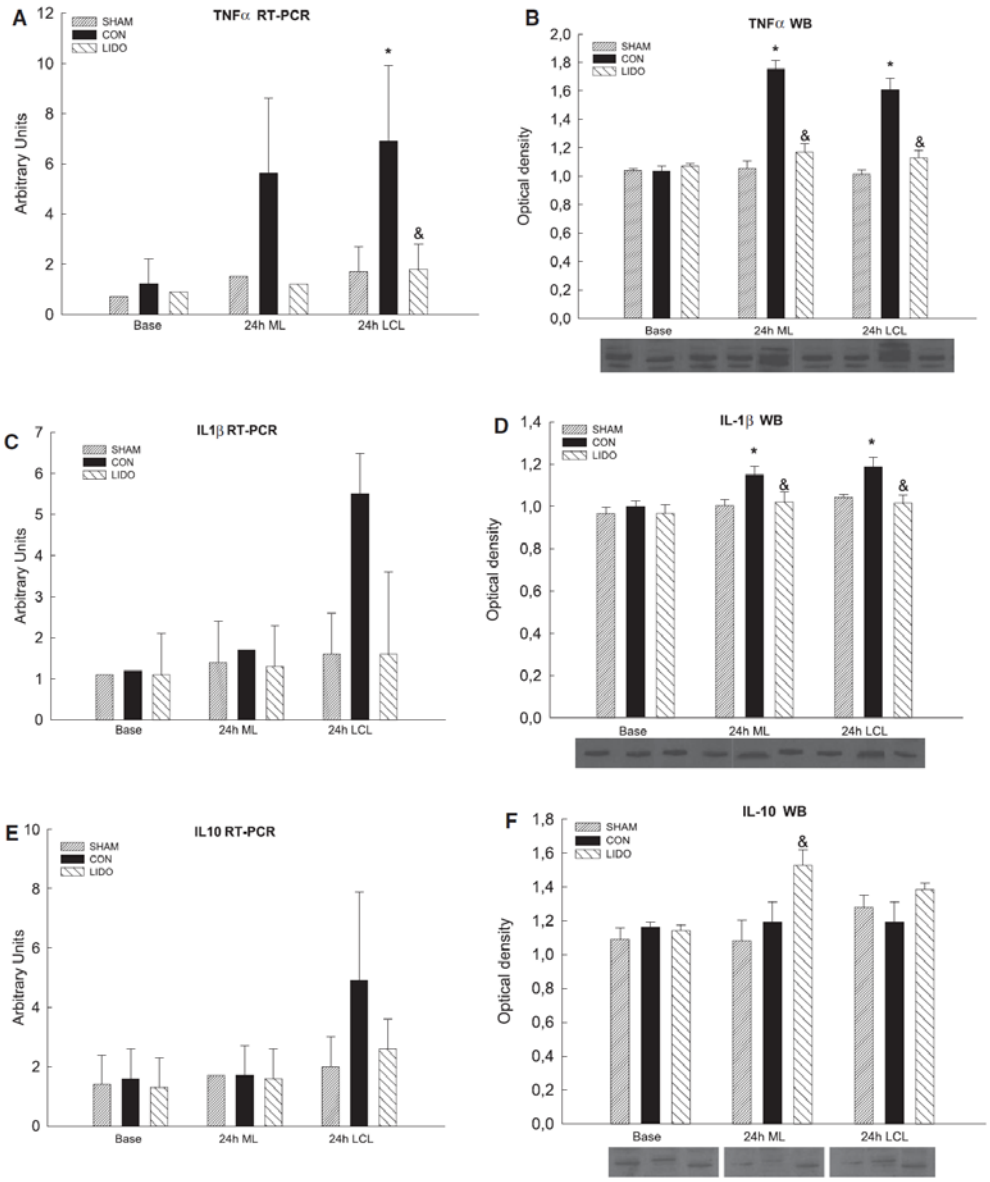

Figure 4. (*Continued*)

decrease in expression of eNOS leads to greater activation of iNOS. Several studies have shown that lidocaine inhibits production of iNOS in macrophages.[36,37] In contrast, eNOS has protective antiinflammatory effects in the vascular endothelium by reducing the adhesion of neutrophils and inhibiting platelet aggregation. The potential effect of lidocaine on expression of iNOS has not yet been studied. In our study, we observed that IV administration of lidocaine increased expression of eNOS in the lungs, especially in the lobe that experienced ischemia-reperfusion. We think that this was yet another protective mechanism of lidocaine in the lungs. In an experimental rabbit model of ischemia-reperfusion, Sedoris et al.[33] hypothesized that the uncoupling of expression and activity of iNOS with respect to eNOS contribute to the lung damage produced during ischemia-reperfusion. In our study,

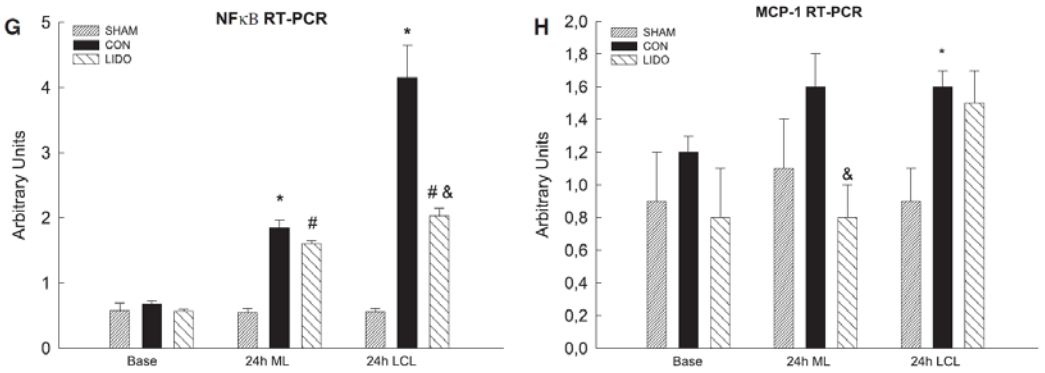

Figure 4. Inflammation in lung biopsies. Bar graphs show the mRNA (RT-PCR) and protein (WB) expressions of inflammatory mediators in lung biopsies and compare the control group (CON) with the lidocaine group (LIDO) and with the SHAM group. Base, lung biopsy of the left caudal lobe taken at the beginning of the surgical procedure; 24-h ML, lung biopsy of the mediastinal lobe (not collapsed during surgery) taken 24 hours after surgery; 24-h left cranial lobe (LCL), lung biopsy of the left cranial lobe (collapsed during surgery) taken 24 hours after surgery. *P < 0.003 CON group versus SHAM group; #P < 0.003 LIDO group versus SHAM group; &P < 0.003 LIDO group versus CON group. (A), mRNA expression of TNF-α; (B) protein expression of TNF-α; (C) mRNA expression of IL-1β; (D) protein expression of IL-1β; (E) mRNA expression of IL-10; (F) protein expression of IL-10; (G) mRNA expression of nuclear factor κB (NFκB); (H) mRNA expression of monocyte chemoattractant protein-1 (MCP-1). TNF = tumor necrosis factor; IL = interleukin; MMP = metalloproteinases.

IV administration of lidocaine, both systemically and locally, maintained stable NOx expression throughout the study, probably because the increase in the proinflammatory isoform iNOS was compensated for by the increase in the pulmonary-protective isoform eNOS. Therefore, the effects of IV lidocaine on the metabolism of NO during LRS also interacted to protect the lungs from damage occurring during this type of surgery.

Apoptosis

Apoptosis is triggered in the presence of an increased inflammatory response (e.g., surgery or MV).[38] Oxidative stress has also been associated with the onset of pulmonary apoptosis,[39] and the forces produced by MV on the lung have been shown to induce apoptosis of pulmonary endothelial cells.[40] Apoptosis is involved in several of the pathophysiological mechanisms underlying ALI, including lesions affecting the alveolar epithelium and regulation of the immune response. Although apoptosis does not generate inflammation, activation of inflammatory pathways (especially NFkB) can increase apoptosis. Depending on the lung cells affected, apoptosis can have beneficial or harmful effects. Thus, apoptosis of neutrophils can prove beneficial in ALI by minimizing release of oxidizing substances and enzymes, whereas apoptosis of alveolar epithelial cells is harmful because it contributes to apoptosis of these cells and the subsequent disruption of the epithelium, leading to ALI. Galani et al.[41] observed that patients with acute respiratory distress syndrome whose BAL specimens showed a more intense apoptotic response have a poorer prognosis. The results of studies on the effect of lidocaine on apoptosis are disparate. Increased apoptosis has been observed in the neutrophils responsible for the inflammatory response.[42] However, studies performed under the condition of ischemia-perfusion–induced injury revealed a clear antiapoptotic effect of lidocaine in the affected tissue, namely a decrease in the area of tissue subject to injury.[43,44]

In our study, we observed a clear antiapoptotic effect of IV lidocaine in lung tissue, both in the lobe affected by ischemia-perfusion and the lobe that was not, probably because high-pressure MV triggers apoptosis in pulmonary epithelial cells.[40] These results suggest that the protective effects of IV lidocaine against ALI induced by ischemia-reperfusion or oxidative stress could be associated with the prevention of epithelial lung cell apoptosis. The mechanisms by which this effect is mediated could be associated with 2 recognized causes of apoptosis: the decrease in TNF-α (and other proinflammatory mediators that we observed) in the BAL specimen, which may be responsible for attenuation of the extrinsic apoptotic pathway, and the inhibition of intrinsic apoptotic pathways, which is represented by the increased expression of the Bcl-2 antiapoptotic protein family and the decreased expression of caspase 9. Caspase 3 is ultimately responsible for the apoptotic effects, and in this study, we observed an attenuation of its expression in the lungs of the LIDO group animals.

Clinical Relevance and Concerns

The potential effects of IV lidocaine on hemodynamic values and arterial oxygenation in routine clinical practice give cause for concern. Our results show that when lidocaine is administered at doses that are considered safe in clinical practice, it does not alter hemodynamic values or affect arterial oxygenation during OLV or within 24 hours of LRS. Therefore, it can be considered safe for clinical practice.

Limitations

Our study is subject to a series of limitations. First, the lidocaine concentration in plasma has not been measured. Doses similar to those used in other studies[44] were chosen; therefore, it can be assumed that lidocaine values were below 2 to 3 μg/mL. Second, analysis of the biomarkers beyond 24 hours could have provided more data; however, since the most significant molecular changes in the ALI occur early, it

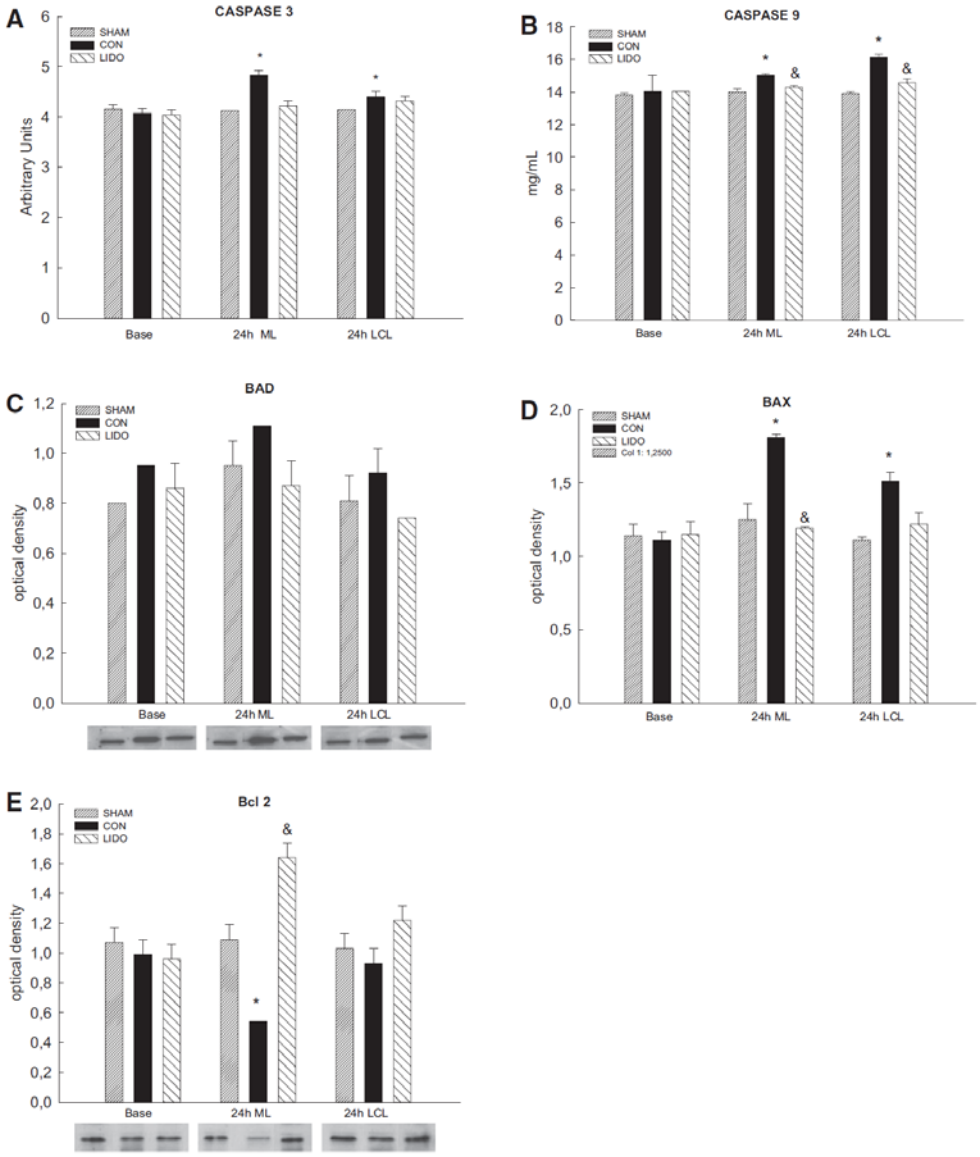

Figure 5. Apoptosis in lung biopsies. Bar graphs show the expression of apoptotic mediators in lung biopsies and compare the control group (CON) with the lidocaine group (LIDO) and with the SHAM group. Base, lung biopsy of the left caudal lobe taken at the beginning of the surgical procedure; 24-h ML, lung biopsy of the mediastinal lobe (not collapsed during surgery) taken 24 hours after surgery; 24-h left cranial lobe (LCL), lung biopsy of the left cranial lobe (collapsed during surgery) taken 24 hours after surgery. *$P < 0.003$ CON group versus SHAM group; #$P < 0.003$ LIDO group versus SHAM group; &$P < 0.003$ LIDO group versus CON group. (A), caspase 3; (B) caspase 9; (C) BAD; (D) BAX; (E) Bcl-2.

is possible that the main results of our study would be confirmed by long time-point samples. Nevertheless, clinical consequences (arterial oxygenation, airway pressure) would probably have been more easily detected on the second or third day after surgery, which is when the most pronounced symptoms appear; therefore, we did not observe them in our study. Last, it is unknown whether these results are reproducible in patients with lungs damaged by smoking or in whom the immune response is altered by the presence of neoplastic disease,[45] in other words, the type of patient

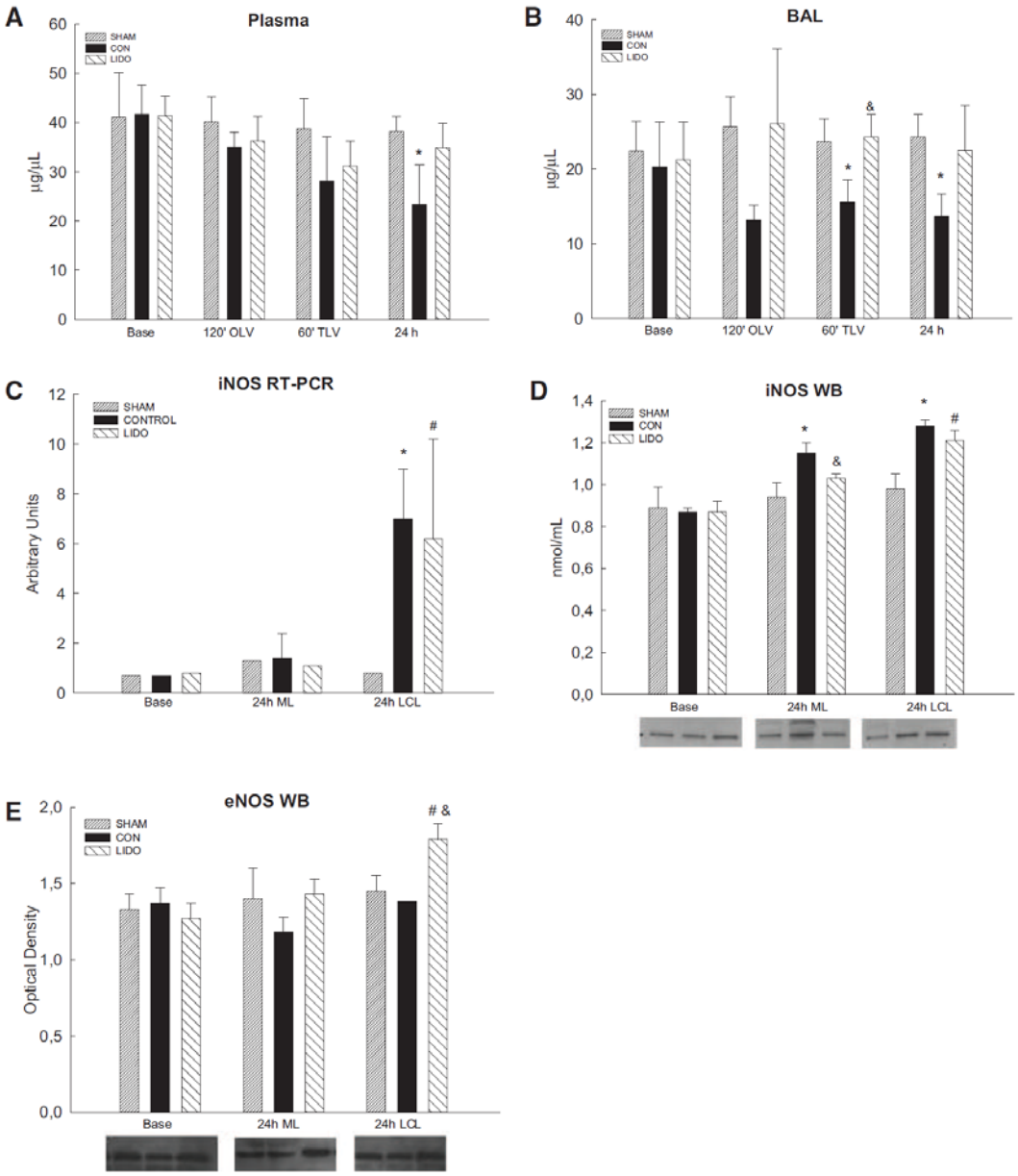

Figure 6. Nitric oxide (NO) metabolism. Bar graphs show the mRNA (RT-PCR) and protein (WB) expressions of nitric oxide metabolism comparing the control group (CON) with the lidocaine group (LIDO) and with the SHAM group. In bronchoalveolar lavage (BAL) and plasma figures: Base, Baseline; 120′ one-lung ventilation (OLV), 120 minutes after the beginning of one-lung ventilation; 60′ two-lung ventilation (TLV), 60 minutes after the beginning of two-lung ventilation; 24 h, 24 hours after surgery. In lung biopsy figures: Base, lung biopsy of the left caudal lobe taken at the beginning of the surgical procedure; 24-h ML, lung biopsy of the mediastinal lobe (not collapsed during surgery) taken 24 hours after surgery; 24-h left cranial lobe (LCL), lung biopsy of the left cranial lobe (collapsed during surgery) taken 24 hours after surgery. *$P < 0.003$ CON group versus SHAM group; #$P < 0.003$ LIDO group versus SHAM group; &$P < 0.003$ LIDO group versus CON group. (A), Nitric oxide metabolites (NOx) in BAL; (B) NOx in plasma samples; (C) mRNA expression of inducible nitric oxide synthase (iNOS) in lung biopsies; (D) protein expression of iNOS in lung biopsies; (E) protein expression of eNOS in lung biopsies. RT-PCR = reverse transcription polymerase chain reaction.

we treat in our daily practice. Further investigation on perioperative management with lidocaine in human patients undergoing LRS is required.

CONCLUSIONS

The results of our porcine model showed that administration of IV lidocaine during LRS attenuated the inflammatory

response (local and systemic) and exerted an antiapoptotic effect in lung tissue 24 hours after LRS. Therefore, we think that this drug could prove to be a novel, safe, and easy-to-use tool for mitigating lung damage that is usually observed after LRS.

DISCLOSURES

Name: Ignacio Garutti, PhD, MD.
Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.
Attestation: Ignacio Garutti examined the original study data, reviewed the data analysis, and approved the final manuscript and is the author responsible for archiving the study files.
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Contribution: This author helped conduct the study, analyze the data, and write the manuscript.
Attestation: Lisa Rancan examined the original study data, reviewed the analysis of the data, and approved the final manuscript and is the author responsible for archiving the study files.
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Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.
Attestation: Carlos Simón examined the original study data, reviewed the analysis of the data, and approved the final manuscript and is the author responsible for archiving the study files.
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Attestation: Gabriel Cusati examined the original study data, reviewed the analysis of the data, and approved the final manuscript.
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Contribution: This author helped conduct the study and write the manuscript.
Attestation: Guillermo Sanchez-Pedrosa examined the original study data, reviewed the data analysis, and approved the final manuscript.
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Attestation: Francisco Moraga examined the original study data, reviewed the data analysis, and approved the final manuscript.
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Contribution: This author helped analyze the data and write the manuscript.
Attestation: Luis Olmedilla examined the original study data, reviewed the data analysis, and approved the final manuscript.
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Contribution: This author helped analyze the data and write the manuscript.
Attestation: Maria Teresa Lopez-Gil examined the original study data, reviewed the data analysis, and approved the final version of the manuscript.
Name: Elena Vara, PhD, MD.
Contribution: This author helped design the study, conduct the study, analyze the data, and write the manuscript.
Attestation: Elena Vara examined the original study data, reviewed the data analysis, and approved the final version of the manuscript.
This manuscript was handled by: Marcel E. Durieux, MD, PhD.

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ARTÍCULO IV

Lidocaine administration controls microRNAs alterations observed after lung ischemia reperfusion injury.

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Subject: MS # ALN201411076 - Decision Letter
Attachment(s): Revision_Due.ics

RE: MS #ALN201411076 - MicroRNA Biomarkers for Detection of Lung Ischemia Reperfusion Injury

Dear Miss Rancan:

Dear Dr Rancan,

Your work has been reviewed by two experts in the field. The subject of the paper is important and relevant, and the findings of interest.

The reviewers raise significant concerns that preclude acceptance of this work in its current form. We are interested in potentially publishing your paper, but clarification regarding the reviewers' comments -and additional new data- would be needed. In addition to responding to all of the reviewers' comments, I make the following points:

#1 Evidence of cause/effect of the role of the chosen miRNAs in attenuation of reperfusion injury.
#2 Evidence of impact of Lidocaine on lessening lung injury (are PaO2 and EVLW taken as markers of injury?) beyond report on inflammatory mediators.
#3 Rationale for choice of the specific miRNAs chosen.
#4 Clarification (cogent argument or new interventional data) of the role of miRNAs in apoptosis in this model.

Please address these concerns in your revision. Also provide a Response to Reviewers Letter indicating where in the manuscript (page, paragraph, and line number) the changes have been made. If you disagree with any of the reviewers' suggestions, please explain your point of view in the same letter. **Distinguish your changes by using a different color. Please do not use the track changes feature.**

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We look forward to receiving a revised paper and responses to the reviewers' comments.

Regards,

Brian P. Kavanagh, M.B., B.Sc., M.R.C.P., F.C.A.R.C.S.I.
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Anesthesiology

MicroRNA Biomarkers for Detection of lung ischemia reperfusion injury

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Abstract:	Background: Ischemia reperfusion injury (IRI) is associated with significant morbidity and mortality. MicroRNAs (miRNAs) have emerged as regulators of IRI and they are involved in the pathogenesis of organ rejection. Lidocaine has proven anti-inflammatory activity in several tissues but its modulation of miRNAs has not been investigated. This work aims to investigate a potential involvement of miRNAs in lung IRI in a lung auto-transplantation model and to investigate the effect of lidocaine. Methods: 3 groups (sham-operated, control and lido), each of 6 large-white pigs, were submitted to a lung auto-transplantation. All groups received the same anesthesia. In addition, animals of lido group received a continuous IV administration of lidocaine (1.5 mg/kg/h) during surgery. Lung tissue samples were taken before pulmonary artery clamp, before reperfusion, 30 minutes post-reperfusion and 60 minutes post-reperfusion. Samples were analysed for different miRNAs (miR-122, miR-145, miR-146a, miR-182, miR-107, miR-192, miR-16, miR-21, miR-126, miR-127, miR142-5p, miR152, miR155, miR-223 and let7) using RT-QPCR. Results were normalized using miR-103. Results: The results showed that the expression of miR-127 and miR-16 did not increase after IRI. All the other miRNAs exhibited a significant increase 60 minutes post-reperfusion. Lidocaine administration significantly reduced the alterations of miRNAs levels observed after lung IRI. Conclusions: The present work demonstrates that lung IRI affects the expression of twelve out of the fourteen tested miRNAs and that the administration of lidocaine reduces significantly these alterations.

Lidocaine administration controls microRNAs alterations observed after lung ischemia reperfusion injury

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Running title: lidocaine alters miRNAs in lung ischemia reperfusion injury

Keywords: miRNAs, lidocaine, ischemia reperfusion injury, lung

Abstract

Background: Ischemia reperfusion injury (IRI) is associated with morbidity and mortality. MicroRNAs (miRNAs) have emerged as regulators of IRI and they are involved in the pathogenesis of organ rejection. Lidocaine has proven anti-inflammatory activity in several tissues but its modulation of miRNAs has not been investigated. This work aims to investigate the involvement of miRNAs in lung IRI in a lung auto-transplantation model and to investigate the effect of lidocaine.

Methods: 3 groups (sham-operated, control and lido), each of 6 pigs, underwent a lung auto-transplantation. All groups received the same anesthesia. In addition, animals of lido group received a continuous IV administration of lidocaine (1.5 mg/kg/h) during surgery. Lung biopses were taken before pulmonary artery clamp, before reperfusion, 30 minutes post-reperfusion (Rp-30) and 60 minutes post-reperfusion (Rp-60). Samples were analyzed for different miRNAs (miR-122, miR-145, miR-146a, miR-182, miR-107, miR-192, miR-16, miR-21, miR-126, miR-127, miR142-5p, miR152, miR155, miR-223 and let7) using RT-QPCR. Results were normalized using miR-103.

Results: The expression of miR-127 and miR-16 did not increase after IRI. Let-7d, miR-21, miR-107, miR-126, miR-145, miR-146a, miR-182 and miR-192 significantly increased at the Rp-60 (control vs. sham-operated $p<0.001$). miR-142-5p, miR-152, miR-155 and miR 223 significantly

increased at the Rp-30 (control vs. sham-operated $p<0.05$) and at the Rp-60 (control vs. sham-operated $p<0.01$). The administration of lidocaine was able to attenuate these alterations in a significant way (control vs lido $p<0.05$).

Conclusions: Lung IRI caused miRNAs dysregulation. The administration of lidocaine reduced significantly miRNAs alterations.

Introduction

Ischemia-reperfusion-induced lung injury (IRLI) has been identified as one of the main causes of primary graft failure. It is characterized by non-specific alveolar damage, lung edema and hypoxemia occurring within 72 hours from the lung transplantation. Despite refinements in lung preservation and improvements in surgical techniques and perioperative care, IRLI remains a significant cause of early morbidity and mortality after lung transplantation 1. Nowadays, although the number of patients on the waiting list is constantly increasing, only 10 to 30% of the donor lungs are used for transplantation 2. Hence, the critical importance of donor organ quality in determining short and long-term graft function is becoming increasingly clear and the identification of new biomarkers to assess the donor lungs is becoming mandatory.

In recent decades, proteomic and genomic analyses have demonstrated that different cell processes and mediators intervene before the disease becomes evident. In this regard, microRNAs (miRNAs), short RNA sequences that act as post-transcriptional regulators which bind themselves to complementary sequences in the 3′ UTR of multiple target messenger RNAs (mRNAs), have emerged as promising disease biomarkers. In fact, recent studies have examined the possibility that changes in miRNAs expression could be used as biomarkers for ischemia-reperfusion injury (IRI) 3. Moreover, it has been observed that some miRNAs are able to regulate the expression of gene targets in the context of lung transplantation 4-6.

It has been observed that local anesthetics, in addition to blocking the inhibitory effects of the nerve signal, have systemic anti-inflammatory properties 7, which have proven to be beneficial in different types of surgeries 8. Among them, lidocaine inhibits migration of polymorphonuclear cells toward the inflamed area by diminishing their mobility and adhesion, with the resulting attenuation in the expression of cytokines and free oxygen radicals 7, 9. These properties have also been observed in relation to lung resection surgery 10; however, little has been reported about the relationship between miRNAs and local anesthetics.

We hypothesized that miRNAs play an active role in the pathogenesis of the IRLI and that this can be reflected by alterations of their levels that can be observed in local samples. In addition, we hypothesized that the administration of lidocaine could modify the expression of miRNAs. Hence, the aim of this study was to investigate a potential involvement of different miRNAs in an IRLI

caused by a left caudal lobe lung auto-transplant model and to investigate the effect of lidocaine administration.

Materials & Methods

The authors declare that the present study has be conducted with the approval of the Committee for Research and Animal Experimentation of Gregorio Marañón General University Hospital (Madrid, Spain), the institution where the animals have be handled. It abides by the provisions of Spanish current legislation in terms of basic standards for the protection and care of animals used in experiments and it is in accordance with the European directive on the protection of animals used for scientific purposes (2010/63/EU).

Animal Model and Study Groups

Eighteen pigs (Sus scrofa) with a weight of 35±7 kg were subjected to an orthotopic left caudal lobe lung transplantation (left pneumonectomy, ex situ cranial lobectomy, and left caudal lobe reimplantation) with a subsequent 60-min graft reperfusion. The anesthetic 10 and surgical 11, 12 procedures have been previously described; the only difference introduced in this work has been that the reperfusion time was set at 60 minutes instead of 30 minutes. Using Excel for PC (Microsoft Corp, Seattle, Washington, USA), pigs were randomly assigned to three groups (6 animals per group): a lidocaine group (LIDO), a control group (CON), and a SHAM group. In animals of the LIDO group, lidocaine was administered as an initial bolus of 1.5 mg/kg followed by a continuous infusion of 1.5 mg/kg/h, which was maintained until the end of the procedure, whereas animals of the CON group received the same volume of 0.9% saline solution. The content of each syringe and infusion was administered blind.

Measurement and sampling time points

Lung biopsies, hemodynamic arterial gas measurements and blood samples were collected at the following time points: Pre-clamping (PreClamp)- before clamping the pulmonary artery; pre-reperfusion (PreRep) - before reperfusion and ventilation of the reimplanted left caudal lobe; 30 min post-reperfusion (Rp-30) - 30 min after the reperfusion of the reimplanted lobe; and 60 min post-reperfusion (Rp-60) - 60 min after the reperfusion of the reimplanted lobe. In addition, hemodynamic arterial gas measurements were collected at the very beginning of the procedure and these values are reported as Base.

Haemodynamic measurements

After the induction, both femoral vein and artery were catheterized, via inguinal cut down, with Arrow-Howes 7 Fr. triple-lumen catheter and PiCCO thermodilution catheter (PV2014L16 Femoral artery in small adults Ø 4F, length 16 cm) respectively. Main hemodynamic variables, fluid responsiveness, preload, after load, contractility and pulmonary oedema were measured by using PiCCO-Pulsion thermodilution monitor. Blood-gas analyses were performed at the beginning of the surgical process (Base); before clamping the pulmonary artery (PreClamp); before starting the reperfusion (PreRep); 30 min and 60 min after reperfusion (30 min Rp and 60 min Rp respectively).

Biochemical studies in lung tissue

Lung tissue biopsies were performed for biochemical studies. Every lung sample was placed in a cryotube, flash-frozen in liquid nitrogen and stored at -80 ºC until biochemical analysis.

Western blotting analysis

Western blots were used to measure the protein expression of TNF-α, IL-1β, Bcl-2-associated death promoter (BAD), Bcl-2-associated X protein (BAX) and caspase 3 as previously described 13. Four samples from each time point of each group were analyzed.

MicroRNAs extraction, quantification and determination

miRNA was isolated from five independent lung samples using the mirVana miRNA Isolation Kit (Ambion, Life Technologies, Texas, USA), following the Enrichment Procedure for Small RNAs according to the manufacturer's instructions. RNA purity and concentration were evaluated by spectrophotometry using µL Biodrop (Isogen Life Science, De Meern, Netherlands) at 260/280 nm (ratio > 2.0). In addition, a 40% acrylamide gel electrophoresis was run to verify the purity as suggested by the mirVana miRNA Isolation Kit protocol's instructions. According to the manufacturer's instructions, the reverse transcription of 350-1000 ng of miRNA was performed using TaqMan® MicroRNA Reverse Transcription Kit for Custom Reverse Transcription Pools with TaqMan® MicroRNA Assay (Ambion, Life Technologies, Texas, USA). RT-PCR was performed using an Applied Biosystems 7300 apparatus with the TaqMan® Universal Master Mix II (Applied Biosystems, Warrington, UK) and 1 µL of the corresponding 20X MicroRNA Assay. For the normalization of cDNA loading in the PCR, the amplification of miR 103 for every sample was used 14. Relative changes in gene expression were calculated using the 2-ΔΔCT method 15.

Statistical analysis ELENA

Sample size was based on previous experience and on literature16. Results are expressed as the mean ± the standard error of the mean. Mean comparison was done by the Kuskal-Wallis test

followed by the Mann Withney test; a confidence level of 95% ($p<0.05$) was considered significant. The SPSS version 14.0 statistical package was used in this study.

Results

General and cardiorespiratory parameters

No differences were observed between the SHAM, CON, and LIDO groups in terms of weight (33.6 ± 2.19 kg; 34.17 ± 3.6 kg; and 39.14 ± 6.36 kg, respectively) or duration of the procedure (275.71 ± 26.99 min; 374.5 ± 10.37 min and 320.71 ± 10.58 min respectively) and between CON and LIDO groups in the duration of the ischemic period (123.3 ± 8.16 min and 127.5 ± 8.89 min respectively). Hemodynamic values, blood gas values, and airway pressures remained very stable during the procedure (Table 1 and 2). The only significant difference was observed between the SHAM group and the other two groups about the PaO2 at the Rp-60 time point and it was presumably caused by the different number of ventilated lungs (Table 2).

miRNAs expression

miRs significantly increased 60 min after reperfusion (Figure 1): A group of miRNAs (let-7d, miR-21, miR-107, miR-126, miR-145, miR-146a, miR-182 and miR-192) exhibited a significantly increased expression at the Rp-60 time point (CON vs. SHAM $p<0.001$). The administration of lidocaine was able to attenuate these alterations in a significant way (CON vs. LIDO $p<0.001$).

miRs significantly increased 30 and 60 min after reperfusion (Figure 2): A group of miRNAs (miR-142-5p, miR-152, miR-155 and miR 223) exhibited an increased expression at the Rp-30 time point (CON vs. SHAM $p<0.05$). Their expression increased even more at the Rp-60 time point (CON vs. SHAM $p<0.01$). miR-152, miR-155 and miR-223 levels of CON group were significantly higher than SHAM group values even before reperfusion (CON vs. SHAM $p<0.05$) suggesting that ischemic injury per se is able to alter their expression. The administration of lidocaine was able to attenuate these alterations in a significant way (CON vs LIDO $p<0.05$). However, unlike the previous group of miRNAs, the LIDO group expressions of miR-142-5p and miR-155 at Rp-60 time point were significantly higher than SHAM group values ($p<0.05$).

miRs with no significant differences (Figure 3): No significant change was observed in the expression of miR-16 and miR-127 inter or intra groups.

Inflammatory markers

A significantly increased expression of TNF-α and IL-1β, two pro-inflammatory cytokines, was observed in the CON group at Rp-30 and Rp-60 time points compared with SHAM and LIDO groups (CON vs. SHAM $p<0.05$). At the Rp-60 time point, it was evident by a significantly increased expression of both cytokines also in LIDO group compared to SHAM group values (LIDO vs. SHAM $p<0.05$). However, these values were significantly lower than those of CON group (CON vs. LIDO $p<0.05$) (Figure 4).

Apoptotic markers

The pro-apoptotic molecules BAD and BAX resulted increased in the CON group at Rp-60 compared with SHAM group (CON vs. SHAM $p<0.05$). BAX levels of CON group were significantly increased also at Rp-30 (CON vs. SHAM $p<0.05$). In the LIDO group, an increased expression of BAD and BAX levels was observed at Rp-60 (LIDO vs. SHAM $p<0.05$). However, this rise was significantly lower than the one observed in the CON group (CON vs. LIDO $p<0.05$).

Discussion

MiRNAs represent a complex mechanism of genetic regulation that can modulate many physiological and pathological processes. Several studies have observed their involvement in the regulation of IRI 17, pulmonary diseases 18, 19, and solid organ transplantation 20. However, to our knowledge, this is the first study on the miRNAs expression in relation to the IRLI induced by lung transplantation. In this study, it has been observed that the IRI derived by lung surgery causes important changes in the expression of several miRNAs. We have observed that some miRNAs were involved in the early phase of reperfusion (miR- 142-5p, miR-152, miR-155 and miR-223) whereas others miRNAs (miR-21, miR-107, miR-126, miR-145, miR-146a, miR-182, miR-192 and let-7d) showed an increased expression only 60 minutes after reperfusion reflecting that their activation occurs later. Thus, our results suggest that miRNAs play a modulating role of the inflammatory response of both early and delayed reperfusion phases. This is in accordance with previous studies that observed increased levels of miRNAs like miR-21 21-24 and miR-223 25 in IRI affecting organs other than lungs.

The inflammatory response is a key stone of the IRI and several miRNAs regulate 26 and are regulated 27 by it. The up regulation of miR-126 has been correlated with the up regulation of TOM1, involved in the IL-1β and TNF-α signaling pathway in cystic fibrosis 28. The deregulation of miR-155 and miR-223 resulted in uncontrolled lung inflammation in different murine models 29. In addition, miR-21 and miR-223 can promote granulocyte differentiation and activation 30. The neutrophil activation and infiltration is an important component of the IRLI 1 and increased MPO levels have been observed in this experiment 30 and 60 minutes after reperfusion in the control group samples (unpublished results). Hence, the increased levels of miR-21 and miR-223 observed in this study could be caused by activated neutrophils (leukocyte-derived miRNAs).

On the other hand, miR-21, miR-145, miR-146a and miR-155, are up regulated by NFκB and/or by inflammatory cytokines like TNF-α or IL-1β 31-34. Moreover, their effects on the inflammatory response can be opposite: miR-155 has pro-inflammatory effects 35-37 whereas miR-146a has an anti-inflammatory effects 38, 39. In our study, the overexpression of all the above mentioned miRNAs was observed which suggests their implication in the inflammatory response caused by IRLI. Since some of them have pro-inflammatory, others have anti-inflammatory effects and some, like miR-145 can be both pro-inflammatory 40 or anti-inflammatory 41, we thick that, in their complex, these miRNAs act like lung-protective, anti-inflammatory miRNAs. However, an improved understanding of the miRNAs target prediction and of how multiple miRNAs collaborate to properly balance the inflammatory response is needed in order to clarify which role is played by each miRNA.

Apoptosis peaks rapidly after reperfusion and it can be induced by oxygen species and inflammatory cytokines 1 or can be activated by an intrinsic pathway. However, in both cases, the effector caspase 3 is expressed. In this study we have observed an increased expression of caspase 3 60 minutes after reperfusion. In addition, at the same time-point, an increased expression of BAD and BAX, two pro-apoptotic molecules of the Bcl-2 family, was observed. Hence, our results confirmed, as previously observed 10 that the IRLI activates apoptotic markers and that this activation involves also the intrinsic pathway. The role of miRNAs in apoptosis regulation is currently under intensive investigation. Recent studies have shown that the overexpression of miR-145 40, 41 and miR-192 42 can cause apoptosis. In our study, an overexpression of miR-145 and miR-192 was observed after reperfusion. At the same time-points, the pro-apoptotic markers were increased too. In addiction, miR-182 and miR-155, both increased after IRI, were predicted to target caspase 3 whereas miR-145, also increased after IRI, has DR4 and DR5 as predicted target 29. Hence, our results suggest that miR-145 and miR-192 could play a role in the activation of pro-apoptotic pathways after IRLI.

Previous studies have observed decreased levels of miR-16 43 and miR-127 44 in case of acute pulmonary lesion and pulmonary edema 45. However, in our study, no significant change was observed in the expression of both miR-16 and miR-127. Hence, our results suggest that these miRNAs do not play a regulating role in the pathogenesis of the IRLI.

The anti-inflammatory properties of lidocaine have been previously described 10 but little is known about its effects on miRNAs expression. In an in vitro model, Sung et al. observed that lidocaine

affected the miRNAs expression on adipose stem cells, but its effect was evident only on few miRNAs 46. In our study, the IV administration of lidocaine affected significantly the expression of all the miRNAs altered by the IRLI. In particular, it canceled or reduced dramatically the alterations observed in the control group. At the same time, lidocaine reduced the expression of pro-inflammatory cytokines and pro-apoptotic markers. To our knowledge, this is the first study regarding the effects of lidocaine administration on miRNAs expression in an in vivo IRLI model.

Different possibilities have been suggested in order to normalize the results of miRs quantification using qPCR. Gu et al. proposed the use of more than one miR as endogenous controls so to have trustworthy results 14. Based on this reference, miR-107 and miR-103 have been picked as endogenous controls; however, whereas miR-103 showed great stability, the expression of miR-107 was significantly increased at Rp-60. Ezzie et al. have observed that miR-107, as well as other members of the miR-15/107 family, were increased in COPD patients 47. Moreover, miR-107 up-regulation has been associated with chronic allograft dysfunction 48. Hence, since this experiment observed the alterations caused by transplantation on the lung tissue, it is not surprising to find increased levels of miR-107. In any case, our results demonstrate that miR-107 cannot be considered as a suitable endogenous control in our porcine experimental model.

Our study is subject to a series of limitations. First, the lidocaine concentration in plasma has not been measured. Doses similar to those used in other studies 49 have been chosen; therefore, it can be assumed that lidocaine values were below 2-3 µg/mL. Second, analysis of the biomarkers beyond 60 min could have provided more data; however, since the most significant molecular changes in the acute lung injury occur early, it is reasonable to consider that the main results of our study may be confirmed by long time-point samples. Nevertheless, the clinical consequences (arterial oxygenation, airway pressure) may probably have been more easily detected on the second or third day after surgery, which is when the most pronounced symptoms appear; therefore, we did not observe them in our study. Third, regarding the translational relevance of this paper, an important limitation is the fact that IRI and organoprotective signaling are largely influenced by common comorbidities and co-medications.

Our study presents also some technical limitations. First, only few miRNAs have been measured in this study. To select the miRNAs presented in this study, we focused on the miRNAs that had been previously described in relation to ischemia reperfusion injury 17, solid organ transplantation 20 and lung pathologies 18, 19, 50. However, not all the miRNAs previously described in relation to these pathologies have been investigated in this study. We consider that a screening using microarray, nanostring or deep sequencing would provide more information about how many and which miRNAs are involved in the IRLI. Second, our study lucks of mechanistic insights into

miRNAs involved in IRLI and the potential links between the changes of miRNAs expression and anti-inflammation conferred by lidocaine. Further studies are required in order to clarify this point.

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Table 1: Hemodynamic parameters

		Base	PreClamp	PreRep	30 min Rp	60 min Rp
HR (bpm)	**SHAM**	99.43±8.01	93.14±7.86	89.71±6.86	93.29±6.71	97.14±5.24
	CON	87.67±5.19	98.16±5.74	104.67±4.6	103.33±5.68	97.33±7.37
	Lido	87.43±5.84	91.14±6.86	78.14±11.79	75.43±13.07	75.14±13.25
MAP (mmHg)	**SHAM**	95.43±11.15	95±10.68	101.43±8.7	98.29±10.22	98.57±8.75
	CON	91±6.9	94.67±7.1	91.67±4.55	78.17±8.53	76.67±5.19
	Lido	93.86±5.46	86.43±3.97	81.71±6.89	60.14±10.94	62.43±12.01
CVP (mmHg)	**SHAM**	8.29±1.67	6±1.53	6.86±1.96	7.43±1.77	8.14±1.92
	CON	7.5±1.3	7±1.35	5±1.39	5±1.22	4.83±1.27
	Lido	7.29±1.04	8.43±1.84	8±1.43	6.29±1.81	6.43±1.86
CI (Lxmin-1xm-2)	**SHAM**	2.96±0.38	2.99±0.2	3.21±0.31	3.13±0.32	3.17±0.39
	CON	3.1±0.19	3.71±0.54	3.86±0.31	3.71±0.26	3.58±0.31
	Lido	3.61±0.49	3.86±0.34	4.79±0.91	2.97±0.55	2.85±0.59
GEDVI (Lxmin-1xm-2)	**SHAM**	613.6±106.7	661.4±109.3	605±86.6	605.4±116.5	583.9±105.5
	CON	500±31.3	534.7±65	529.7±27.3	512.5±38.7	522.8±83.7
	Lido	747.7±168.7	587.9±70.7	674.6±125.9	359.4±83.2	431.7±150.2
DPMAX (mmHg/s)	**SHAM**	562.8±94.8	611±87.5	622.6±89.3	604.6±71.3	662.4±104.1
	CON	515±56.25	488.3±78.4	563.3±48.3	513.3±55.8	486.7±57.9
	Lido	477.3±85.4	710.1±67.8	563.4±54.5	501±94.1	425.7±127.4
ELWI	**SHAM**	8.17±1.25	7.5±1.41	8.83±4.92	7±1.04	6.67±0.85
	CON	10.67±1.82	9.67±1.68	9.17±2.21	8.67±1.58	7±0.53
	Lido	13±4.03	11.33±4.1	9.33±3.32	5.17±1.16	6±1.4
SVV (%)	**SHAM**	15.86±1.06	8.29±2.36	9.71±2.81	10.71±3.59	8.43±3.31
	CON	12.83±3.66	14.33±7.06	3.58±7.86	5.12±6	5.47±7.29
	Lido	16.29±5.12	11±3.42	7.86±3.44	6±2.89	7.29±6.6
SVRI (dyn/s/cm-5/m2)	**SHAM**	2560.2±369	2232.3±349	2099.3±264	2146.5±174	2344.2±239
	CON	2046.8±165	2105±260.2	1797±182	1734.8±198	1439.3±189
	Lido	2042.6±265	1803.6±238	1505.1±244	1524.7±125	1504.8±178

Data are expressed as the mean ± SEM. HR, heart rate; MAP, mean arterial pressure; CVP, central venous pressure; CI, cardiac index; GEDVI, global end-diastolic volume; DPMAX, maximum diastolic pressure; ELWI, extra-vascular lung water; SVV, stroke volume variation; SVRI, systemic vascular resistance index.

CON, control group; LIDO, lidocaine group; SHAM, sham group. Base, basal value; PreClamp, value obtained before clamping the pulmonary artery; PreRep, value obtained before starting the reperfusion; 30 min Rp and 60 min Rp, values obtained 30 min and 60 min after reperfusion respectively.

Table 2: Blood gases and respiratory parameters

		Base	PreClamp	PreRep	30 min Rp	60 min Rp
SaO2 (%)	**SHAM**	100±0	99.86±0.38	100±0	99.86±0.38	99.57±1.13
	CON	100±0	98.17±4.49	100±0	98.33±2.88	98.17±2.99
	Lido	100±0	99.86±0.38	99.29±1.5	100±0	100±0
PaO2 (mmHg)	**SHAM**	305.57±36.67	215.57±54.44	251±52.56	237.29±66.56	195.86±53.5
	CON	287.83±65.7	210.17±59.41	205.83±45.51	259±54.95	279.33±49.1*
	Lido	360.86±106.32	262.14±137.04	205.43±78.05	311.83±86.23	310.25±40.8*
PaCO2 (mmHg)	**SHAM**	41±6.22	43±7.39	44±8.54	45±9.71	46.14±9.55
	CON	44.83±14.52	41.6±5.59	37.17±4.62	46.2±9.31	43.83±9.08
	Lido	41.57±9.8	42.33±2.94	39.5±6.65	40.17±6.85	37.67±7
pH	**SHAM**	7.5±0.04	7.49±0.04	7.49±0.05	7.48±0.07	7.48±0.06
	CON	7.49±0.12	7.47±0.14	7.54±0.06	7.47±0.09	7.49±0.08
	Lido	7.52±0.1	7.48±0.08	7.48±0.14	7.52±0.06	7.54±0.07
PpAW (cmH2O)	**SHAM**	27.38±5.53	26.29±4.1	26.09±3.62	27.4±3.58	29.33±3.2
	CON	32.83±3.48	34.2±2.83	33.5±3.66	37±2.67	35.8±3.02
	Lido	24.5±3.32	27.71±3.73	27.43±3.24	25.17±3.58	30±3.34
Mean P (cm H2O)	**SHAM**	7.64±4.94	9.04±4.45	8.3±3.81	8.87±4.32	8.78±3.35
	CON	7.5±1.52	7.8±1.3	8.8±1.3	8.6±2.07	8.4±1.14
	Lido	6.96±0.76	7.71±0.95	8.7±1.6	7.5±1.05	8±0.63

Data are expressed as the mean ± standard error of the mean. SaO_2, oxygen saturation; PaO_2, partial pressure of oxygen in arterial blood; $PaCO_2$, partial pressure of carbon dioxide in arterial blood; PpAW, pulmonary artery wedge pressure; mean P, mean pressure.

CON, control group; LIDO, lidocaine group; SHAM, sham group. Base, basal value; PreClamp, value obtained before clamping the pulmonary artery; PreRep, value obtained before starting the reperfusion; 30 min Rp and 60 min Rp, values obtained 30 min and 60 min after reperfusion respectively. *$p<0.05$ vs. SHAM group

Table 3: Schematic view of surgical procedure, anaesthetic protocol and sampling points

Samples time-point		Base	PreClamp (before clamping the pulmonary artery)			PreRep (before reperfusion of the left caudal lobe)	30 min Rp and 60 min Rp (respectively 30 and 60 mins after the reperfusion of the reimplanted lobe
Anaesthetic protocol	Lido	PROPOFOL + IV LIDOCAINE ADMINISTRATION					
	Control	PROPOFOL					
	SHam	PROPOFOL					
	Number of ventilated lungs	TLV		OLV			TLV
	Surgical procedure	Sedation	Left Thoracotomy	Left pneumonectomy (except Sham group)	Left cranial lobectomy *ex situ* (except sham group)	Left caudal lobe implantation (except Sham group)	Repefusion

Figure Legends

Figure 1: Bar graphs show the expression of different miRNAs (**A**, let-7d; **B**, miR-21; **C**, miR-107; **D**, miR-126; **E**, miR-145; **F**, miR-146a; **G**, miR-182; **H**, miR-192) in lung samples throughout the experiment and compare the control group (CON) with the lidocaine group (LIDO) and with the SHAM group.

PreClamp, value obtained before clamping the pulmonary artery; PreRep, value obtained before starting the reperfusion; Rp-30 and Rp-60, values obtained 30 min and 60 min after reperfusion respectively.

*p < 0.001 CON group vs. all.

Figure 2: Bar graphs show the expression of different miRNAs (**A**, miR-142-5p; **B**, miR-152; **C**, miR-155; **D**, miR-223) of lung samples throughout the experiment and compare the control group (CON) with the lidocaine group (LIDO) and with the SHAM group.

PreClamp, value obtained before clamping the pulmonary artery; PreRep, value obtained before starting the reperfusion; Rp-30 and Rp-60, values obtained 30 min and 60 min after reperfusion respectively.

*P < 0.05 CON group vs. SHAM group, LIDO group and CON PreClamp. *P<0.001 vs. all. # P<0.05 LIDO group vs. SHAM group.

Figure 3: Bar graphs show the expression of different miRNAs (**A**, miR-16; **B**, miR-127) of lung samples throughout the experiment and compare the control group (CON) with the lidocaine group (LIDO) and with the SHAM group.

PreClamp, value obtained before clamping the pulmonary artery; PreRep, value obtained before starting the reperfusion; Rp-30 and Rp-60, values obtained 30 min and 60 min after reperfusion respectively.

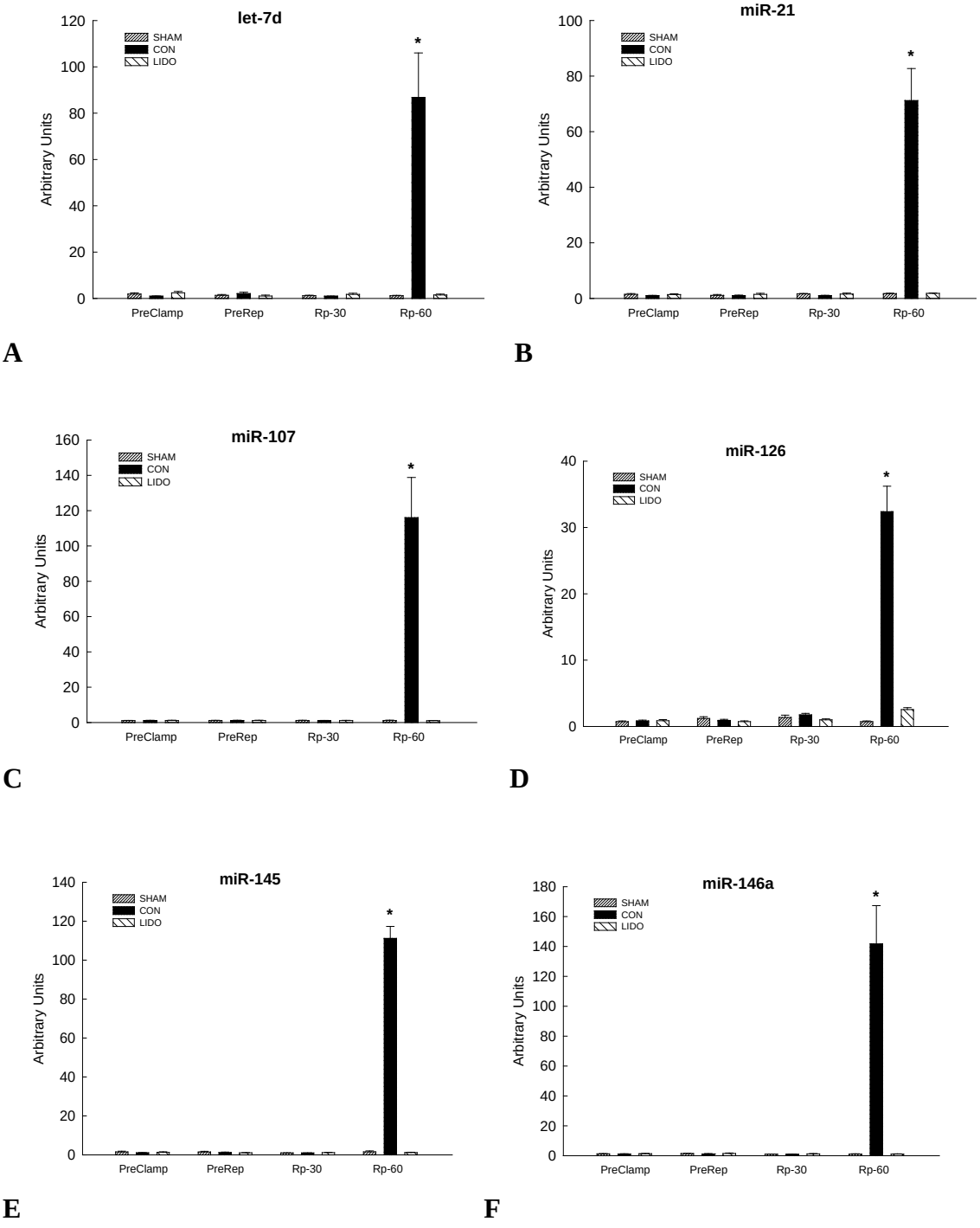
Figure 4: Bar graphs show the protein expression of inflammatory mediators in lung biopsies and compare the control group (CON) with the lidocaine group (LIDO) and with the SHAM group.

PreClamp, value obtained before clamping the pulmonary artery; PreRep, value obtained before starting the reperfusion; Rp-30 and Rp-60, values obtained 30 min and 60 min after reperfusion respectively.

*p<0.005 vs. SHAM and LIDO groups; #p<0.05 vs. SHAM group; **p<0.001 vs. all.

A, protein expression of TNFα; **B,** protein expression of IL-1β; **C,** Bcl-2-associated death promoter (BAD); **D,** Bcl-2-associated X protein (BAX) and **E,** caspase 3

Figure 1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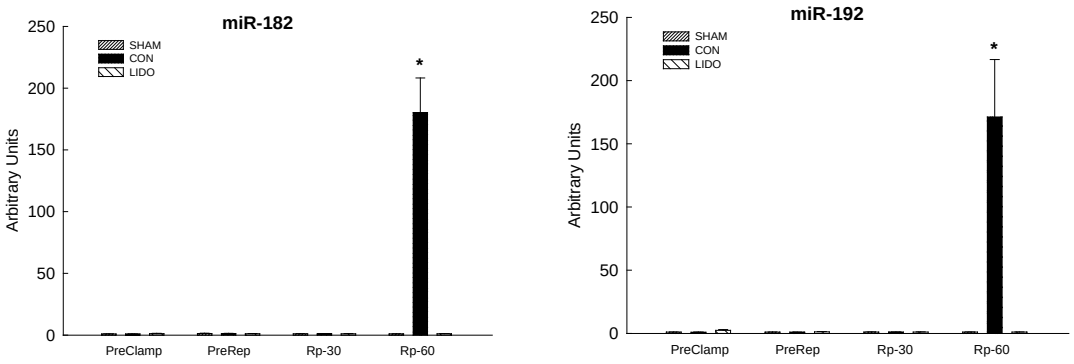
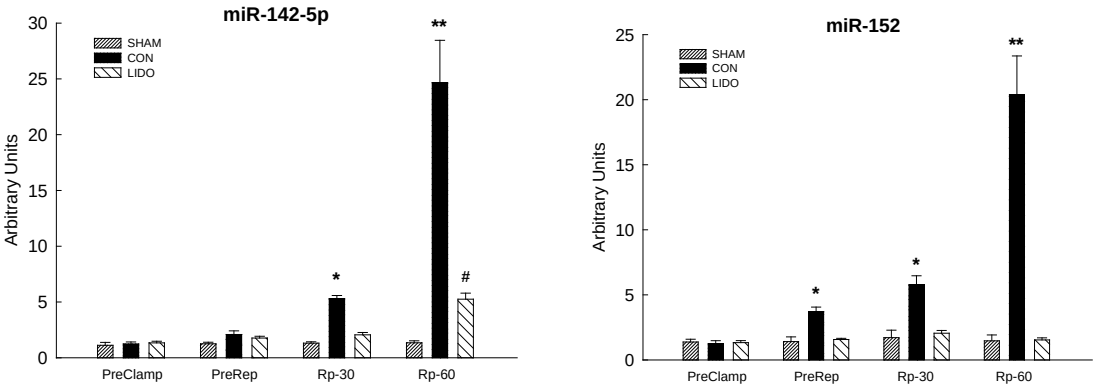
miR-21
miR-107
miR-126
miR-145
miR-146a
SHAM
CON
LIDO
Arbitrary Units
PreClamp
PreRep
Rp-30
Rp-60
*
A
B
C
D
E
F

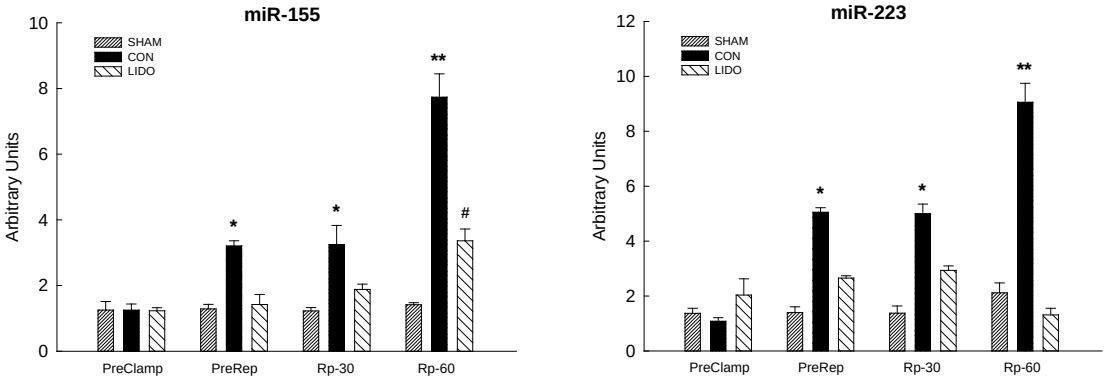


G **H**

Figure 2

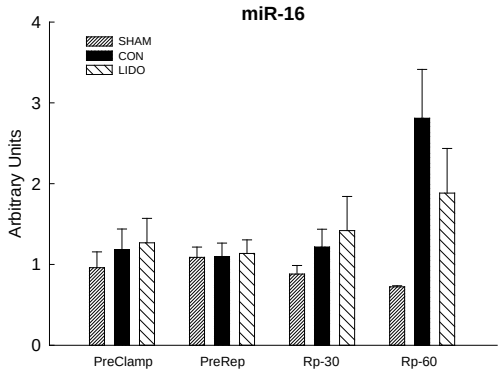


A **B**

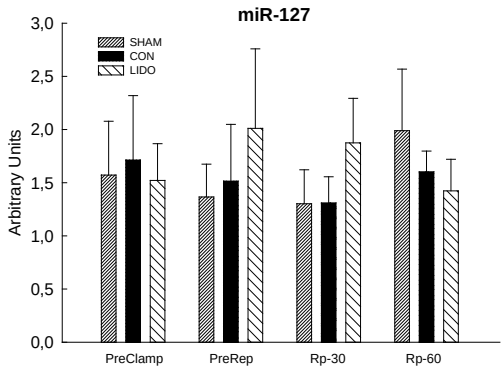


C **D**

Figure 3

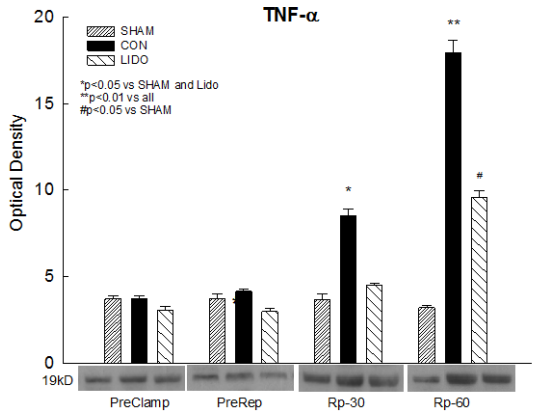


A

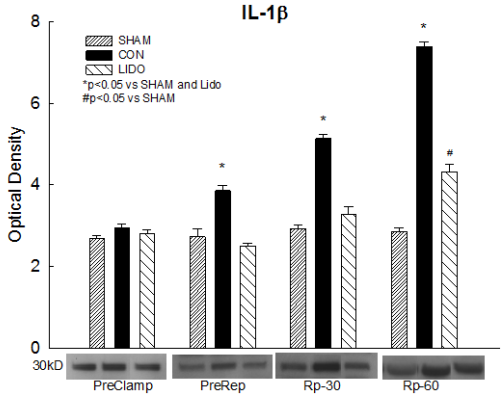


B

Figure 4



A



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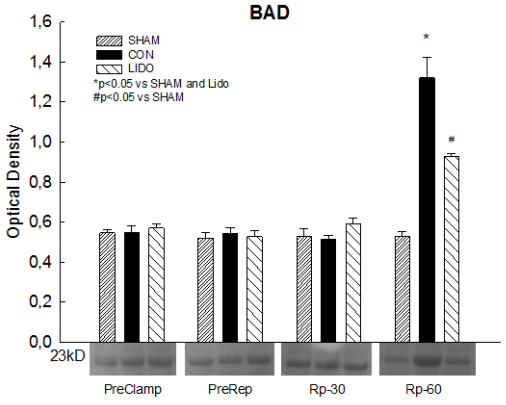

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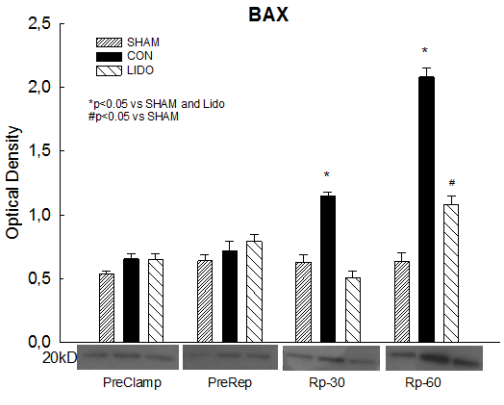
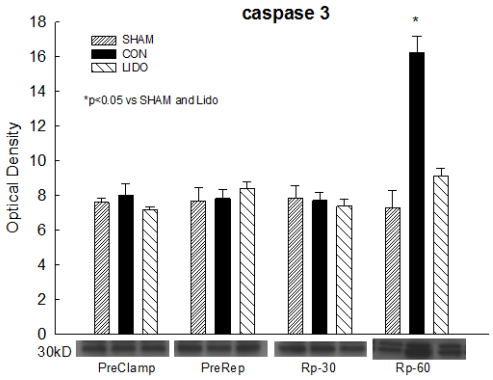


D


caspase 3
SHAM
CON
LIDO
*p<0.05 vs SHAM and Lido
Optical Density
18
16
14
12
10
8
6
4
2
0
*
30kD
PreClamp
PreRep
Rp-30
Rp-60

E

ARTÍCULO V

Glycocalyx degradation after pulmonary resection surgery

Lisa Rancan, Guillermo Sánchez-Pedrosa, Karen Aymonnier, Javier Casanova, Celia Muñoz, David Rincón, Carlos Simón, Ignacio Garutti, Elena Vara.

British Journal of Anaesthesia

Decision Letter (BJA-2015-00099-HG015)

From: h.f.galley@abdn.ac.uk

To: lisaranc@ucm.es

CC:

Subject: British Journal of Anaesthesia - BJA-2015-00099-HG015

Body: Dear Ms. Rancan,

BJA-2015-00099-HG015
Glycocalyx degradation after pulmonary resection surgery

I have now received our expert Assessors' Reports on the manuscript that you submitted for publication in the British Journal of Anaesthesia.

These reports are given below. You will see that the assessors have raised several problems regarding your manuscript. If you feel you are able to modify your manuscript in the light of these criticisms, I shall be pleased to consider a revised manuscript.

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Prof. Helen Galley

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Glycocalyx degradation after pulmonary resection surgery

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Key Words:	Anaesthetics local - lidocaine, Surgery - thoracic, glycocalyx

Glycocalyx degradation after pulmonary resection surgery

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Running title: Glycocalyx degradation after pulmonary resection

Keywords: Anaesthetics local – lidocaine, surgery – thoracic, glycocalyx

Abstract

Background: Ischaemia-reperfusion injury (IRI) is a main cause of morbidity after pulmonary resection surgeries. The dysfunction of glycocalyx, a dynamic layer of macromolecules at the luminal surface of the endothelium, seems to participate in tissue dysfunction after IRI. Lidocaine has proven anti-inflammatory activity in several tissues but its modulation of glycocalyx has not been investigated. This work aims to investigate a potential involvement of glycocalyx in lung IRI in a lung auto-transplantation model and to investigate the effect of lidocaine.

Methods: 3 groups (sham-operated, control and lidocaine), each of 6 large-white pigs, were submitted to a lung auto-transplantation. All groups received the same anaesthesia. In addition, animals of lido group received a continuous IV administration of lidocaine (1.5 mg/kg/h). Lung tissue and plasma samples were taken before pulmonary artery clamp, before reperfusion, 30 minutes post-reperfusion and 60 minutes post-reperfusion in order to analyse pulmonary oedema, glycocalyx components, adhesion molecules, myeloperoxidase, nitric oxide (NO) and endothelial nitric oxide synthase (eNOS).

Results: The ischaemia caused pulmonary oedema that was greater after reperfusion when decreased levels of syndecan-1 and heparan sulphate were observed in lung samples whereas increased levels were observed in plasma samples. After reperfusion, neutrophil activation and expression of adhesion molecules were increased whereas NO and eNOS levels were decreased. All these alterations were significantly minor or absent in the lidocaine group.

Conclusions: Lung IRI caused glycocalyx degradation that contributed to neutrophil activation and adhesion. The administration of lidocaine was able to protect the lung from glycocalyx degradation.

Introduction

During several thoracic surgical procedures, the lung experiences an ischaemia/reperfusion (I/R)-induced damage which has been identified as one of the main causes of primary graft failure 1. Although the severity of damage resulting from I/R varies between tissues, a common component of this pathologic process for all organs is microvascular dysfunction 2, 3. In fact, after I/R, endothelial cells suffer from increased oxidative stress 4 and they exhibit swelling and detachment from the basement membrane 5. Consequently, leukocytes adhere and transmigrate 6, 7 and vascular permeability increases 8. These endothelial consequences of I/R suggest an involvement of the endothelial glycocalyx, which is a dynamic layer of macromolecules on the luminal surface of vascular endothelium that is involved in fluid homeostasis and regulation. Within the lung, the damage or degradation of glycocalyx integrity may be a major mediator for oedema formation after lung I/R injury (IRI). Although the pathogenesis of pulmonary oedema and specific aetiologies of lung injury after lung IRI are still to be understood 9, ongoing investigations are improving our

understanding of the complex factors involved in the pathophysiology of post-resection lung injury. However, there is a paucity of data, particularly related to glycocalyx integrity/damage and lung IRI.

Recent studies showed that local anaesthetics, in addition to blocking the inhibitory effects of the nerve signal, have systemic anti-inflammatory properties 10, which have proven to be beneficial in different types of surgeries 11. Among them, lidocaine inhibits migration of polymorphonuclear cells toward the inflamed area by diminishing their mobility and adhesion, with the resulting attenuation in the expression of cytokines and free oxygen radicals 10, 12. These properties have also been observed in relation to lung resection surgery 13; however, to our knowledge, there is paucity of data related to the effects of lidocaine administration on glycocalyx integrity in lung IRI.

Hence, the aim of our work was to investigate a possible injury of the glycocalyx caused by lung IRI after pulmonary resection and also to investigate whether the administration of lidocaine would have been able to reduce this injury.

Methods

The authors declare that the present study has be conducted with the approval of the Committee for Research and Animal Experimentation of Gregorio Marañón General University Hospital (Madrid, Spain), the institution where the animals have been handled. It abides by the provisions of Spanish current legislation in terms of basic standards for the protection and care of animals used in experiments and it is in accordance with the European directive on the protection of animals used for scientific purposes (2010/63/EU).

Animal Model and Study Groups

Eighteen large white pigs with a weight of 35±7 kg were subjected to an orthotopic left caudal lobe lung transplantation (left pneumonectomy, ex situ cranial lobectomy, and left caudal lobe reimplantation) with a subsequent 60-min graft reperfusion. The anaesthetic 13 and surgical 14, 15 procedures have been previously described; the only difference introduced in this work has been that the reperfusion time was set at 30 and 60 min instead of 10 and 30 min. Using Excel for PC (Microsoft Corp, Seattle, Washington, USA), pigs were randomly assigned to three groups (6 animals per group): lidocaine group (LIDO), control group (CON), and SHAM group. In animals

of the LIDO group, lidocaine was administered as an initial bolus of 1.5 mg/kg followed by a continuous infusion of 1.5 mg/kg/h, which was maintained until the end of the procedure, whereas animals of the CON group received the same volume of 0.9% saline solution. The content of each syringe and infusion was administered blind.

Measurement and sampling time points

Lung biopsies and plasma samples were collected at the following time points: Pre-clamping (PPn)- before clamping the pulmonary artery; pre-reperfusion (PRp) - before reperfusion and ventilation of the reimplanted left caudal lobe; 30 min post-reperfusion (Rp-30') - 30 min after the reperfusion of the reimplanted lobe; and 60 min post-reperfusion (Rp-60') - 60 min after the reperfusion of the reimplanted lobe. In addition, haemodynamic arterial gas measurements were collected at the very beginning of the procedure and these values are reported as Base.

Biochemical studies in lung tissue

Lung tissue biopsies were performed for biochemical studies. Each lung and plasma sample was placed in a cryotube, flash-frozen in liquid nitrogen and stored at -80º C until biochemical analysis.

Wet-to-dry ratio – pulmonary oedema

In order to quantify the wet-to-dry ratio, approximately 50 mg of each lung sample was incubated for 12 h at 60° C and weighed again. The values obtained were analysed with the following formula: [wet weight – dry weight] / wet weight.

Enzyme-linked immunosorbent assays (ELISA)

Levels of heparan sulphate, syndecan-1, VCAM and ICAM-1 were measured in plasma and/or lung biopsies samples using porcine specific ELISA kits (Cusabio Biotech Co. Wuhan Hubei, China), following manufacturer's instructions. Briefly, standards and samples were pipetted into pre-coated wells and any of the investigated protein present in the samples was bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for the investigated protein was added to the well. After washing, avidin conjugated horseradish peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells. After 10 min, the colour development was stopped and the intensity of the colour was measured at 450 nm.

Myeloperoxidase determination

The myeloperoxidase (MPO) assay was used to quantitate lung tissue neutrophil accumulation, and it was detected by the modified Bradley method of Bradley et al. 16.

NOx determination

The concentration of nitric oxide (NO) metabolites was measured in blood samples using the Griess test, which determined the concentration of NO2 after reduction of NO3 to NO2. Reproducibility within the assays was evaluated in three independent experiments, and each assay was carried out with three replicates. The overall intra-assay coefficient of variation was calculated to be <5%. Assay-to-assay reproducibility was evaluated in three independent experiments. The overall inter-assay coefficient of variation was calculated to be <6%.

Western blotting

Western blotting was used to measure levels of endothelial nitric oxide synthase (eNOS). Briefly, after homogenization with lysis buffer, tissue samples (50-60 mg) were boiled with gel-loading buffer (0.100 M Tris-Cl; 4% sodium dodecyl sulfate [SDS]; 20% glycerol; 0.1% bromophenol blue) at a 1:1 ratio and sonicated. The protein concentrations were determined using the bicinchoninic acid assay. The total protein equivalents (25 μg) for each sample were separated using SDS-polyacrylamide gel electrophoresis with 10% acrylamide gels and transferred onto a nitrocellulose membrane using a semi-dry transfer system. The membrane was immediately placed in blocking buffer containing 5% non-fat milk in 20 mM Tris (pH 7.5), 150 mM NaCl, and 0.01% Tween-20. The blot was allowed to block at 37° C for 1 hour. The membrane was incubated with anti-rat rabbit polyclonal eNOS (dilution 1:1000) antibody for 12 h at 4° C, followed by incubation with an anti-rabbit horseradish peroxidase-conjugated IgG antibody (1:2000). After washing with T-TBS, the membranes were incubated with ECL Plus detection reagents (Amersham Life Science Inc., Buckinghamshire, UK) and exposed to X-ray film. The films were scanned using a densitometer (BioRad GS 800) to determine the relative optical densities. Pre-stained protein markers were used for molecular weight determinations, and the bands were analysed using Quantity ONE 1.0 Analysis Software, version 4.5.2.

Statistical analysis

Nonparametric tests were used. The Kruskal-Wallis test was used in order to identify any significant difference between the groups. Subsequently the Mann Whitney test was used to analyse the specific sample pairs for significant differences. Statistical significance was set at $p<0.05$. All data were expressed as mean ± standard error of the mean (±SEM). The SPSS version 14.0 statistical package was used in this study.

Results

The ischaemic injury caused oedema in both the CONTROL and the LIDO groups and the injury was greater after the reperfusion. However, the administration of lidocaine significantly reduced the oedema caused by the IRI (Fig. 1). The neutrophils presence also increased after IRI, as observed in the lung tissue (Fig. 2A) and in the plasma samples (Fig. 2B) of the CONTROL group. In the lung, this alteration was observed 30 min after reperfusion and increased even more 60 min after reperfusion whereas in plasma samples it was evident only 60 min after reperfusion. The administration of lidocaine was able to reduce in a significant way the activation of neutrophils after reperfusion. Moreover, no alteration was observed in the plasmatic levels of MPO in the LIDO group (Fig. 2).

The IRI caused a significant reduction of the levels of syndecan-1 in the lung samples of CONTROL group. This effect was evident at both 30 and 60 min after reperfusion (Fig. 3A)

At the same time-points, in the CONTROL group, increased levels of sindecan-1 were observed in the plasma samples (Fig. 3B). The administration of lidocaine significantly counteracted these alterations in both lung and plasma samples (Fig. 3). These results suggest that the lung IRI causes a damage of the glycocalyx. Integrity of the glycocalyx is compromised and, thus, increased levels of the major component of the glycocalyx, syndecan-1, are observed in plasma samples. In accordance with this fact, increased levels of heparan sulphate, another important component of the glycocalyx, were significantly increased in plasma samples of the CONTROL group 60 min after reperfusion. Once again, the administration of lidocaine was able to reduce this alteration (Fig. 4).

The IRI also increased the expression of adhesion molecules as observed after reperfusion in the CONTROL group (Fig. 5). Alterations due to IRI were observed analysing both the levels of VCAM in the lung tissue (Fig. 5A) and the levels of ICAM-1 in the plasma samples (Fig. 5B). Once again, the administration of lidocaine significantly reduced these changes (Fig. 5).

The ischaemic injury caused a reduction of the plasmatic NO expression as observed in the CONTROL group before reperfusion. This effect was maintained also after reperfusion (Fig.6A). In addition, at the same time-points, in the CONTROL group it was observed a significantly decreased expression of eNOS (Fig. 6B). These alterations were absent in the LIDO group, where the only significant decrease was observed in the levels of NO before the reperfusion (Fig. 6A).

Discussion

One of the main factors that contribute to lung injury after pulmonary resection surgeries is I/R. Even if the pathogenesis of IRI is still to be completely understood, ongoing investigations suggest that the degradation of the endothelial glycocalyx may play a key role in it 17-19. Accordingly, our results showed that also the IRI that affects the lung after a lung resection surgery compromises the glycocalyx integrity. This damage can be observed in lung samples, where both syndecan-1 and heparan sulphate levels were decreased after reperfusion, and in the plasma samples, where they increased at the same time-points. Similar results have been previously observed in a heart model, where the inflammatory response induced glycocalyx damage 20. Our results demonstrate that also after lung resection surgeries the endothelial glycocalyx is damaged. In our study, glycocalyx degradation was detected also in plasma samples, which has a great interest in clinical scenarios. Our results are in accordance with previous studies that observed glycocalyx degradation in plasma samples of adults undergoing aortic surgery 21.

Frequently, the increased oxidative stress and the inflammatory response activated during the ischaemia cause pulmonary oedema, which was also observed in our study at the PRp time-point. A damage of the glycocalyx integrity causes a loss of cellular junctions and the function of endothelial barrier of the glycocalyx disappears, thus promoting pulmonary oedema formation. In our study, it was observed that, after reperfusion, the oedema was greater than the one observed at the PPn time-point. Glycocalyx degradation was also observed after reperfusion, which suggests that the glycocalyx injury could contribute to create the oedema observed in our experimental model.

Another consequence of the glycocalyx dysfunction is the expression of adhesion molecules and the consequent activation of neutrophils. On the other hand, neutrophil adhesion to altered endothelial cells is facilitated by augmented expression of adhesion molecules, activated by pressure-mediated changes 22. In our study, after reperfusion, an increased expression of adhesion molecules was evident both in lung and in plasma samples. At the same time-points, also neutrophil activation, measured as MPO expression was observed. Even though further studies would be required in order to clarify the relation between neutrophil activation and glycocalyx dysfunction, our results clarify that both of them are an important part of the IRI pathogenesis.

Glycocalyx is also important in mechanotransduction; particularly, glycocalyx is the mechanosensor for the NO response, activating eNOS and causing increased vascular permeability.

During increased vascular pressure, the increased hydraulic flow through the glycocalyx deforms or stresses the glycosaminoglycan fibres, which in turn activate eNOS and leads to barrier dysfunction18. In our study, both NO and eNOS showed a decrease when glycocalyx was damaged and an oedema was evident. Even if our results seem to be in contrast with the effect of glycocalyx on the NO production, we consider the main explanation has to be found in the time of reperfusion. Once that IRI occurs, it causes vasoconstriction. In this scenario, it is observed a drop in eNOS expression and NO production in early reperfusion phase. In our results, in accordance with previous studies with similar reperfusion time-points 23, 24, lower expressions of both NO and eNOS were observed. We suppose that longer reperfusion times my show additional alterations of NO metabolism.

Considering the role of the endothelial glycocalyx in maintaining the homeostasis, preventing its shedding or degradation may have important clinical benefits during thoracic surgeries. In this regard, in this study, it has been observed that the administration of lidocaine is able to significantly reduce the alterations of the glycocalyx caused by IRI. Our results show that lidocaine can also reduce the lung oedema, the expression of adhesion molecules and the alterations observed in the NO metabolism.

The inflammatory response, and particularly the production of pro-inflammatory cytokines, can intensify the glycocalyx degradation 18 and it has been previously observed that lidocaine has important anti-inflammatory effects in the IRI observed after pulmonary resection surgeries 25, 26. Hence, it is possible that the protective effect of lidocaine on glycocalyx may be due to its anti-inflammatory properties. Further studies are required in order to understand the exact pathway that leads to glycocalyx protection mediated by lidocaine administration.

From a clinical point of view, it is interesting that both the glycocalyx degradation and the effectiveness of lidocaine administration can be detected in plasma samples, allowing the clinicians to monitor in a quick and low-invasive way the lung injury caused by IRI after pulmonary resection surgery.

MicroRNAs (miRs), short RNA sequences that act as post-transcriptional regulators, have emerged as promising disease biomarkers. In fact, recent studies have examined the possibility that changes in miRNAs expression could be used as biomarkers for IRI 27. In a recent study conducted by our research group, increased expressions of several miRs were observed after lung IRI (Rancan L, personal communication, 2014); among others, miR-126 showed an increase. Interestingly, this miR targets syndecan-1 and VCAM. Further studies should be able to clarify whether the degradation of the glycocalyx is modulated also by the miRs expression in the lung IRI.

The main limitation of our study is the short reperfusion time; the analysis of the biomarkers beyond 60 min of reperfusion could have provided more data. However, since the most significant

molecular changes in the acute lung injury occur at an early stage, it is reasonable to consider that the main results of our study may be confirmed by long time-point samples.

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Authors' contribution:

Lisa Rancan, This author helped conduct the study, analysed the data, and wrote the manuscript. Lisa Rancan examined the original study data, reviewed the data analysis and approved the final manuscript. She is the author responsible for archiving the study files.

Guillermo Sanchez-Pedrosa, This author helped conduct the study and wrote the manuscript. Guillermo Sanchez-Pedrosa examined the original study data, reviewed the data analysis, and approved the final manuscript.

Karen Aymonnier, This author helped conduct the study and wrote the manuscript. Karen Aymonnier examined the original study data, reviewed the data analysis, and approved the final manuscript.

Javier Casanova, This author helped design the study, conduct the study and wrote the manuscript. Dr. Javier Casanova examined the original study data, reviewed the data analysis, and approved the final manuscript.

Celia Muñoz, This author helped conduct the study and analysed the data. Celia Muñoz reviewed the data analysis and approved the final manuscript.

David Rincón, This author helped conduct the study and analysed the data. David Rincón reviewed the data analysis and approved the final manuscript.

Carlos Simón, This author helped design the study, conducted the study and analysed the data. Dr. Carlos Simón examined the original study data, reviewed the data analysis and approved the final manuscript.

Ignacio Garutti, This author helped design the study, conducted the study and analysed the data. Dr. Ignacio Garutti reviewed the data analysis, and approved the final manuscript.

Elena Vara, This author helped design the study, conducted the study, analysed the data, and wrote the manuscript. Dr. Elena Vara examined the original study data, reviewed the data analysis and approved the final manuscript. She is the author responsible for archiving the study files.

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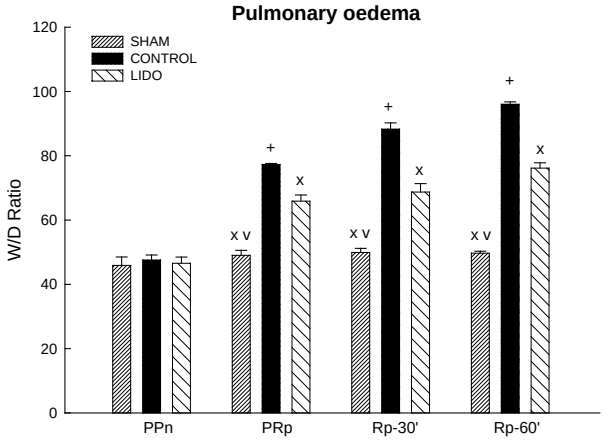
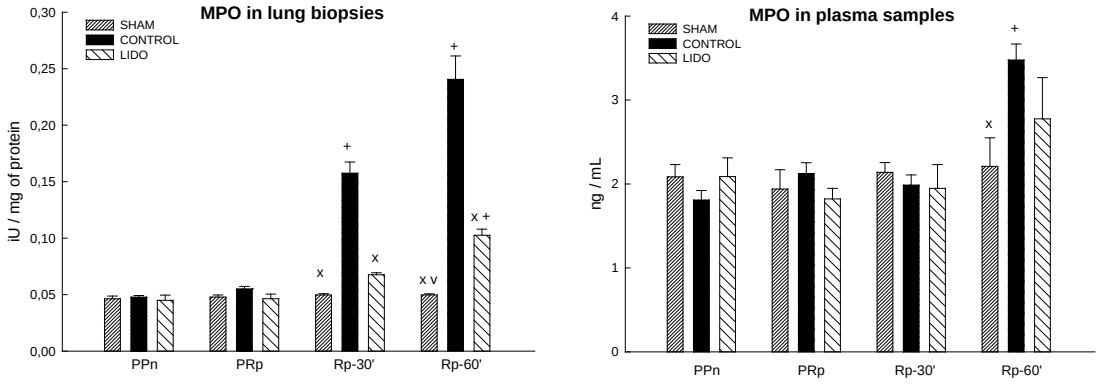
Figure 1



The bar graphs show the expression of the pulmonary oedema of lung biopsies comparing the control group with the lidocaine group (LIDO) and with the SHAM group. PPn, lung biopsy taken at the beginning of the surgical procedure; PRp, lung biopsy taken at the end of the surgical procedure, after the ischemic period and before the beginning of the reperfusion; Rp-30', lung biopsy taken 30 min after the beginning of the reperfusion, Rp-60', lung biopsy taken 60 min after the beginning of the reperfusion. +p< 0.05 vs. PPn; Xp<0.05 vs. CONTROL; Vp<0.05 vs. LIDO.

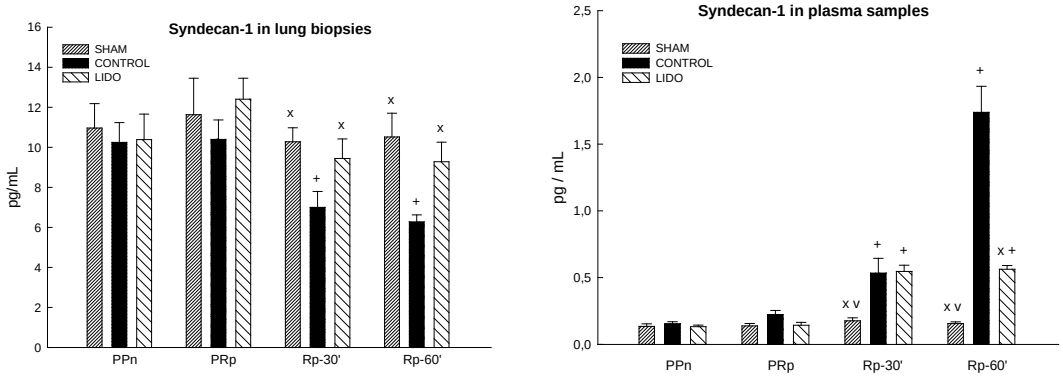
Figure 2



A **B**

The bar graphs show the levels of myeloperoxidase (MPO) in lung biopsies (**A**) and in plasma samples (**B**) comparing the control group with the lidocaine group (LIDO) and with the SHAM group. PPn, lung biopsy taken at the beginning of the surgical procedure; PRp, lung biopsy taken at the end of the surgical procedure, after the ischemic period and before the beginning of the reperfusion; Rp-30', lung biopsy taken 30 min after the beginning of the reperfusion, Rp-60', lung biopsy taken 60 min after the beginning of the reperfusion. +p< 0.05 vs. PPn; **X**p<0.05 vs. CONTROL; **V**p<0.05 vs. LIDO.

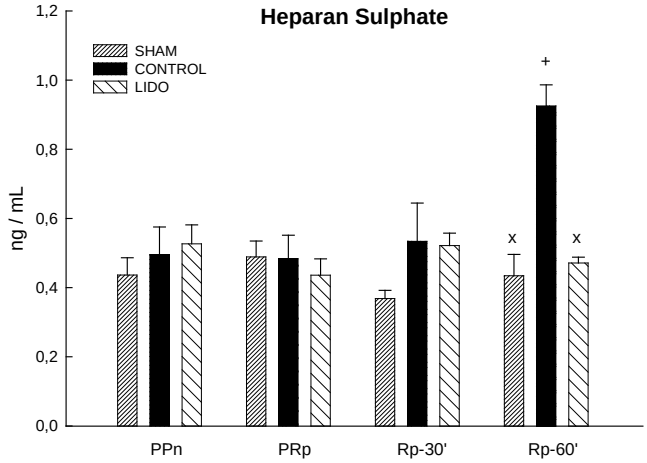
Figure 3



A **B**

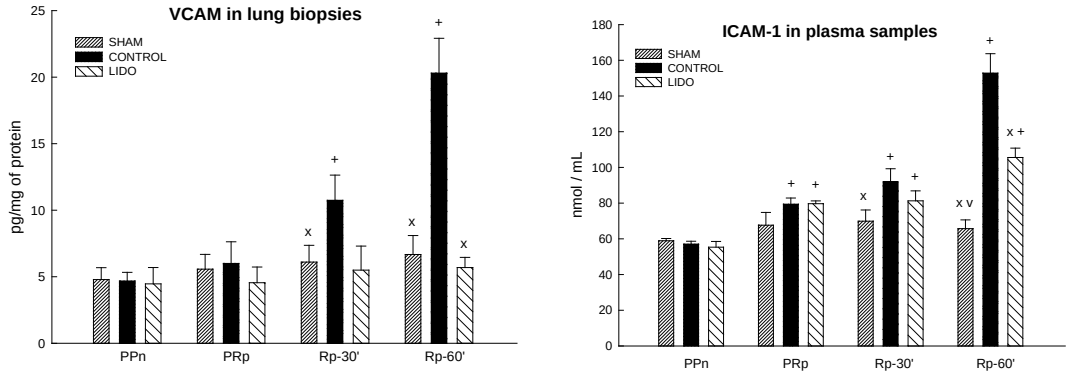
The bar graphs show the levels of syndecan-1 in lung biopsies (**A**) and in plasma samples (**B**) comparing the control group with the lidocaine group (LIDO) and with the SHAM group. PPn, lung biopsy taken at the beginning of the surgical procedure; PRp, lung biopsy taken at the end of the surgical procedure, after the ischemic period and before the beginning of the reperfusion; Rp-30', lung biopsy taken 30 min after the beginning of the reperfusion, Rp-60', lung biopsy taken 60 min after the beginning of the reperfusion. +p< 0.05 vs. PPn; **X**p<0.05 vs. CONTROL; **V**p<0.05 vs. LIDO.

Figure 4



The bar graphs show the levels of heparan sulphate in plasma samples comparing the control group with the lidocaine group (LIDO) and with the SHAM group. PPn, lung biopsy taken at the beginning of the surgical procedure; PRp, lung biopsy taken at the end of the surgical procedure, after the ischemic period and before the beginning of the reperfusion; Rp-30', lung biopsy taken 30 min after the beginning of the reperfusion, Rp-60', lung biopsy taken 60 min after the beginning of the reperfusion. +p< 0.05 vs. PPn; **X**pP 0.05 vs. CONTROL; **V**p<0.05 vs. LIDO.

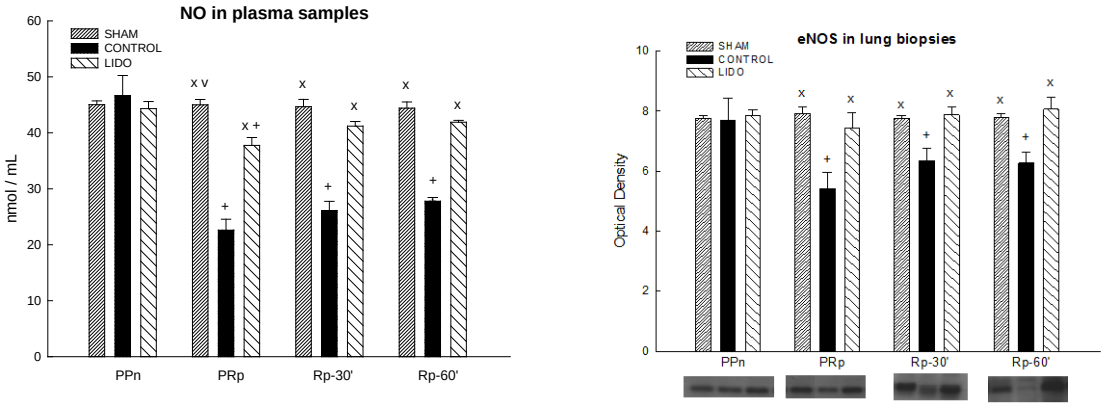
Figure 5



A **B**

The bar graphs show the levels of vascular cell adhesion protein (VCAM) in lung biopsies (**A**) and intercellular adhesion molecule 1 (ICAM-1) in plasma samples (**B**) comparing the control group with the lidocaine group (LIDO) and with the SHAM group. PPn, lung biopsy taken at the beginning of the surgical procedure; PRp, lung biopsy taken at the end of the surgical procedure, after the ischemic period and before the beginning of the reperfusion; Rp-30', lung biopsy taken 30 min after the beginning of the reperfusion, Rp-60', lung biopsy taken 60 min after the beginning of the reperfusion. +p< 0.05 vs. PPn; **X**p<0.05 vs. CONTROL; **V**p<0.05 vs. LIDO.

Figure 6



A **B**

The bar graphs show the levels of nitric oxide (NO) in plasma samples (**A**) and endothelial nitric oxide synthase (eNOS) in lung biopsies (**B**) comparing the control group with the lidocaine group (LIDO) and with the SHAM group. PPn, lung biopsy taken at the beginning of the surgical procedure; PRp, lung biopsy taken at the end of the surgical procedure, after the ischemic period and before the beginning of the reperfusion; Rp-30', lung biopsy taken 30 min after the beginning of the reperfusion, Rp-60', lung biopsy taken 60 min after the beginning of the reperfusion. +p< 0.05 vs. PPn; **X**p<0.05 vs. CONTROL; **V**p<0.05 vs. LIDO.

ARTÍCULO VI

Chemokine involvement in lung injury secondary to ischaemia/reperfusion

Lisa Rancan, David Rincón, Luis Huerta, Sergio D. Paredes, Cruz García, Ignacio Garutti, Federico González-Aragoneses, Carlos Simón, Elena Vara

Chemokine involvement in lung injury secondary to ischaemia/reperfusion

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Running head: Chemokines in lung ischemia-reperfusion injury.

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Abstract word count: 280

Manuscript word count: 2111

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Abstract

Objectives: During transplant surgeries, the lung experiences an ischaemia-reperfusion (I/R) induced damage identified as a significant cause of morbidity and mortality in the early postoperative period. One of the major contributors of this injury is the recruitment of circulatory leukocytes. However, the mechanisms by which I/R induces leukocyte accumulation and subsequent tissue damage in lung surgeries remains unknown. Therefore, the present study aims to assess the role of monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2) in leukocyte chemotaxis related to lung injury secondary to I/R.

Methods: Six pigs were subjected to an orthotopic left caudal lobe lung transplantation with a subsequent 60 minutes graft reperfusion (CON group). In addition, six animals underwent to sham surgery (Sham Group). Plasma samples and lung biopsies were collected before the beginning of pneumonectomy, before starting the reperfusion, 30 minutes after the beginning of the reperfusion and 60 minutes after the beginning of the reperfusion. Plasma levels of intercellular adhesion molecule 1 (ICAM-1) and lung expression of monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 2 (MIP-2), myeloperoxidase (MPO) and lung oedema were measured.

Results: Lung I/R caused substantial damage observed as pulmonary oedema. The oedema was evident after the ischemic insult and increased after reperfusion. After reperfusion,

increased levels of MPO were observed which suggests an activation and infiltration of neutrophils into the lung tissue. After 30 min of reperfusion, MCP-1, MIP-2 and ICAM-1 levels were significantly increased compared to prepneumonectomy levels ($p<0.05$) and a further increase was observed after 60 min of reperfusion ($p<0.05$).

Conclusions: The present study demonstrates that activated neutrophils, as well as MCP-1, MIP-2 and ICAM-1 are involved in inflammatory response induced by ischemia-reperfusion-induced lung injury.

Keywords: ICAM-1, ischemia-reperfusion injury, lung, MCP-2, MIP-1

Introduction

Lung transplantation is currently the only treatment that can prolong the life of patients with terminal lung diseases. Currently, the number of viable organs available is very limited and the selection criteria of the subsidiary lung transplant are stringent, resulting in long waiting lists. For this reason, research is necessary to optimize the lung transplantation process and to face an important issue of healthcare demand. During transplant surgery the lung experiences an ischaemia/reperfusion (I/R)-induced damage which is known to be the consequence of the following elements: apoptosis, oxidative stress and both local and systemic inflammatory response (Carden, Granger 2000). These factors have been shown to lead to lung damage that can compromise the viability of the organ. In fact, ischaemia/reperfusion injury (IRI) is a main cause of primary graft failure (de Perrot et al. 2003). However, the biochemical mechanisms that mediate the inflammatory response in lung transplantation are still to be completely understood. Thus, there is a need for a better

understanding of the cellular and molecular events associated with ischemia-reperfusion-induced- lung injury (IRLI) in order to develop more site-specific interventions that could mitigate inflammatory injury during early reperfusion without interfering with lung healing. One of the major factors of acute inflammation is the recruitment of circulatory leukocytes. Leukocyte accumulation can increase the release of reactive oxygen species and proteases, promoting progressive tissue damage, and contributing to increased vascular permeability (Aghajanian et al. 2008). However, the mechanisms by which I/R induces leukocyte accumulation and subsequent tissue damage in lung surgeries remains unknown.

For this reason, the aim of the present study is to assess the role of monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2) in leukocyte chemotaxis related to lung injury secondary to I/R.

Materials and Methods

This study was approved by the institution's Research and Animal Experimentation Committee. All experiments were performed according to European and Spanish law regarding the handling and care of experimental animals.

Animal Model and Study Groups

Six pigs (*Sus scrofa)*(35 ± 4 kg) were subjected to an orthotopic left caudal lobe lung transplantation (left pneumonectomy, ex situ cranial lobectomy, and left caudal lobe re-implantation) with a subsequent 30 minutes graft reperfusion (CON group). In addition, six animals were submitted to sham surgery (Sham Group).

Anaesthesia

The anaesthetic procedure has been previously described (Garutti et al. 2014). Briefly, the anaesthetic premedication was performed with IM ketamine (10 mg/kg; Ketolar, Parke Davis, Pfizer, Dublin, Ireland). The induction was conducted with propofol (4 mg/kg; Diprivan, AstraZeneca, Macclesfield, Cheshire, UK), fentanyl (3 µg/kg; Fentanest, Kern Pharmaceuticals, Houston, TX), and atracurium (0.6 mg/kg; Tracrium, Glaxo Smith Kline, Brentford, UK). Orotracheal intubation was performed with a 6- to 7-mm cuffed endotracheal tube. Mechanical ventilation with volume-controlled ventilation was used with 5 cm H2O positive end expiratory pressure and peak pressure <30 cm H2O throughout the study. A tidal volume approximately 8 mL/kg, a respiratory rate of 12 to 15 respirations per minute (rpm), and an inspiratory to expiratory ratio of 1:2 were chosen to maintain PaCO2 in the range of 35 to 40 mmHg. FiO2 was maintained at 1 throughout the procedure. Intraoperative crystalloid infusion was maintained at 5 to 6 mL kg-1 h-1. Anaesthesia was maintained with propofol in continuous infusion (8 to 10 mg kg-1 h-1). Supplemental doses of fentanyl and atracurium were used when required.

Surgical protocol

The surgical procedure has been previously described, with the only difference that the reperfusion time was set to 60 minutes instead of 30 minutes (Casanova et al. 2011, Simon Adiego et al. 2011). Briefly, we performed a left thoracotomy and we placed the endotracheal tube into the right bronchus starting in this way the one lung ventilation (OLV). Then the pneumonectomy was performed. Just before the completion of the pneumonectomy, a bolus of IV heparin (300 IU/kg; Mayne Pharma, Madrid, Spain) was

administered to prevent thrombosis in the clamped pulmonary artery. Next, on the back table, the left lung was perfused through the pulmonary artery and veins with University of Wisconsin solution, and a cranial lobectomy was performed to ensure that a thrombus did not form during the graft. The caudal left lobe was implanted back into the swine, and the reperfusion was performed in a retrograde direction by unclamping the left atrium. The endobronchial tube was then pulled back into the trachea, which enabled 2-lung ventilation. The left pulmonary artery was then unclamped, and blood flow was maintained for 60 minutes. At the end of the experiment, the animal was euthanized by a potassium chloride injection while under deep anaesthesia. Animals in the Sham group underwent the same protocol than in the CON group, including thoracotomy, except for lung resection and OLV.

Measurement and sampling time points

Plasma samples and lung biopsies were collected before completing pneumonectomy (PRE-PN), before reperfusion and ventilation of the reimplanted left caudal lobe (PRE-RP); 30 minutes after the beginning of the reperfusion of the reimplanted lobe (30'P-RP) and 60 minutes after the beginning of the reperfusion of the reimplanted lobe (60'P-RP).

Enzyme-linked immunosorbent assays (ELISA)

Plasma levels of intercellular adhesion molecule 1 (ICAM-1) and lung levels of monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2) were measured by using specific commercial ELISA kits according to the manufacturer's instructions (Biosource International Inc., Camarillo, CA, USA).

Wet-to-dry ratio

In order to quantify the wet-to-dry ratio, approximately 50 mg of each lung sample was incubated for 12 hours at 60°C and weighed again. The values obtained were analysed with the following formula: [wet weight – dry weight] / wet weight.

MPO

The myeloperoxidase (MPO) assay was used to quantitate lung tissue neutrophil accumulation, and it was detected by the modified Bradley method of Bradley et al. (Bradley et al. 1982).

Statistical analysis

The data are expressed as the mean and the standard error of the mean (SEM). Nonparametric tests were used. Accordingly, a Mann-Whitney U-test was applied to establish differences between the analysed groups. In addition, a Wilcoxon test for paired data was used to study the evolution of the intragroup values. Statistical significance was considered at $p \leq 0.05$. The SPSS version 14.0 statistical package was used in this study.

Results

Lung I/R caused substantial pulmonary damage determined as lung oedema (Figure 1). This oedema, measured as wet to dry ratio, was accompanied by increased neutrophil accumulation, as measured by tissue MPO content (Figure 2). However, MPO elevation was only observed after reperfusion indicating that oedema is already evident before the accumulation and activation of neutrophils.

After 30 minutes of reperfusion, both MCP-1 and MIP-2 levels were significantly increased compared to PRE-PN levels ($p<0.05$) and a further increase was observed after 60 minutes of reperfusion ($p<0.05$) (Figure 3 and 4 respectively). Similarly, plasma levels of ICAM-1 were significantly increased 30 minutes after reperfusion in CON group compared to PRE-PN levels ($p<0.05$) and a further increase was observed 60 minutes after the beginning of the reperfusion ($p<0.05$) (Figure 5).

Discussion

Ischemic periods of significant duration result in an inflammatory response; this response is both accelerated and augmented when the ischemic tissue is reperfused. The consequent injury has been identified as a significant cause of morbidity and mortality in the early postoperative period. In addition, severe ischemia-reperfusion injury (IRI) has been associated with an increased risk of acute rejection and it is considered as a main cause of primary graft failure (de Perrot et al. 2003). In this study, we have observed that the ischemic insult causes pulmonary oedema and that this injury is greater after reperfusion. The reperfusion injury affecting the lung exhibits a bimodal pattern, consisting of neutrophil-independent events during the first few hours of reperfusion and of neutrophil-mediated events after 4 hours of reperfusion (Eppinger et al. 1995). Neutrophils progressively infiltrate the transplanted lung during the initial 24 hours of reperfusion playing an important role in the inflammatory response (Adoumie et al. 1992). In this study, we have observed increased levels of MPO 30 and 60 minutes after reperfusion. Hence, our results confirm that the reperfusion injury causes neutrophils activation and infiltration within the lung. However, previous studies observed that other leukocytes, such as macrophages, have a more important role in the early phase of reperfusion (Fiser et al. 2001). Macrophages, as well as lymphocytes, produce several chemokines known for their

chemotactic and pro-inflammatory effects. However, their role in IRLI is not fully understood yet. MIP-2 is a chemokine secreted by monocytes and macrophages that is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells. Aratani et al. demonstrated that this chemokine participates in pulmonary inflammation and that it regulates MPO and, as well, can be regulated by it (Aratani et al. 2012). In accordance with that study, our results confirm that MIP-2 expression increased after IRLI. Moreover, in our study, MIP-2 levels increased before reperfusion whereas MPO levels increased after reperfusion, suggesting that macrophages participate in the early phase of reperfusion and that MIP-2 contributes to neutrophils activation in case of IRLI. MIP-2 is not the only chemokine involved in peripheral leukocytes recruitment. There is overwhelming evidence that one of the best-studied CC chemokines, MCP-1, plays a critical role in the development of several acute and chronic diseases; however, the molecular mechanisms underlying the role of MCP-1 in the development and progression of lung IRI caused by lung auto-transplantation remains poorly understood. Several studies have investigated the role of MCP-1 in cardiovascular diseases (Niu, Kolattukudy 2009). Experiments from animal models have shown that myocardial MCP-1 is responsible for mononuclear cell recruitment into the ischemic myocardium (Birdsall et al. 1997) and that it is involved in the inflammatory process associated with transplantation that can lead to transplant vasculopathy and tissue destruction, resulting eventually in a rejection. In fact, early and persistent expression of MCP-1 in cardiac allografts has been implicated in transplant arteriosclerosis and allograft rejection (Horiguchi et al. 2002, Russell et al. 1993, Bharat et al. 2008) and increased levels of MCP-1 have been reported in human myocardial

transplant patients (de Groot-Kruseman et al. 2001, de Groot-Kruseman et al. 2003, Hognestad et al. 2005). In these patients, increased MCP-1 levels were associated with immediate graft dysfunction and rejection. To our knowledge, few investigations have been performed about the role of MCP-1 in the inflammatory process that affects the lung after the ischemia-reperfusion episode (Simon Adiego et al. 2011). In accordance with Simon et al., in this work we have observed increased levels of MCP-1 after reperfusion. Hence, our results confirm that MCP-1 plays an important role in monocyte recruitment during IRLI, as it does in others tissues. MCP-1 has shown some promise as biomarker for disease monitoring in other inflammatory diseases, such as juvenile rheumatoid arthritis (Yao et al. 2006). Thus, the knowledge of its involvement in the lung IRI caused by lung auto-transplantation might open new possibilities for this molecule as diagnostic biomarker in lung surgery.

Another important component of the IRI is the adhesion of leukocytes, since neutrophils accumulation *per se* would not be enough to cause tissue injury. In fact, *in vivo* studies observed that toxic products are almost exclusively secreted by adherent neutrophils (Frangogiannis et al. 2002). ICAM-1 is one of the primary ligands for the CD18 integrins (Albelda et al. 1994); it facilitates both emigration of neutrophils and their adherence-dependent cytotoxic behaviour. ICAM-1 involvement in IRI has been –and still is– object of intensive investigation since it seems to be possible to use it not only for diagnosis, but also for therapy (Tuttolomondo et al. 2009). In our study, increased levels of ICAM-1 were observed after reperfusion. Hence, our results suggest that the neutrophils observed in the lung would be adherent or, at least, that an increased adhesion stimulus was present, implying that it would be the toxic products secreted by the adhered neutrophils that could cause, at least in part, the lung injury observed after reperfusion. Moreover, in this study we decided to analyse ICAM-1 levels in plasma. In fact, this molecule has been previously

investigated as target of treatment of the IRI (Tuttolomondo et al. 2009). Thus, to observe any alteration of its levels in plasma samples would not only be proof of a systemic injury, but would also be helpful to monitor treatment outcome when an anti-inflammatory treatment would be used. In addition, it has been previously observed that newly expressed ICAM-1 may participate in the tissue injury associated with reperfusion only when leukotactic gradient and neutrophil activation are present. TNF-α may be a crucial factor for initiating the cytokine cascade responsible for ICAM-1 induction and subsequent neutrophil-induced injury (Frangogiannis et al. 1998). About lung IRI, previous studies observed an increased expression of cytokines, such as TNF-α among others (de Perrot et al. 2003, Simon Adiego et al. 2011, Casanova et al. 2011, Garutti et al. 2014). Thus, it is likely to suppose that the cytokine cascade associated with the IRI could be the cause, at least in part, of the increased levels of ICAM-1 observed in this study.

Conclusion

The present study demonstrates that adherent and activated neutrophils, as well as MCP-1 and MIP-2, are involved in inflammatory response induced by ischemia-reperfusion-induced lung injury. This information may not only help identifying the molecular pathways involved and improve the understanding of the molecular basis of the IRLI, but it may also provide potential biomarkers for diagnosis and early identification of such condition as well as potential therapeutic targets.

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Conflict of interest: none declared.

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Figure legends

Figure 1: Bar graphs show the expression of oedema, measured as wet to dry ratio, in lung samples throughout the experiment and compare the control group (CON) with the SHAM group.

PRE-PN, value obtained before clamping the pulmonary artery; PRE-RP, value obtained before starting the reperfusion; 30'P-RP and 30'P-RP, values obtained 30 min and 60 min after reperfusion respectively.

*$p < 0.001$ vs. SHAM.

Figure 2: Bar graphs show the expression of MPO in lung samples throughout the experiment and compare the control group (CON) with the SHAM group.

PRE-PN, value obtained before clamping the pulmonary artery; PRE-RP, value obtained before starting the reperfusion; 30'P-RP and 30'P-RP, values obtained 30 min and 60 min after reperfusion respectively.

*$p<0.001$ vs. SHAM.

Figure 3: Bar graphs show the expression of MIP-2 in lung samples throughout the experiment and compare the control group (CON) with the SHAM group.

PRE-PN, value obtained before clamping the pulmonary artery; PRE-RP, value obtained before starting the reperfusion; 30'P-RP and 30'P-RP, values obtained 30 min and 60 min after reperfusion respectively.

*$p<0.05$ vs. SHAM; **$p<0.001$ vs. SHAM

Figure 4: Bar graphs show the protein expression of MCP-1 in lung biopsies and compare the control group (CON) with the SHAM group.

PRE-PN, value obtained before clamping the pulmonary artery; PRE-RP, value obtained before starting the reperfusion; 30'P-RP and 30'P-RP, values obtained 30 min and 60 min after reperfusion respectively.

*p<0.001 vs. SHAM.

Figure 5: Bar graphs show the protein expression of ICAM-1 in plasma samples and compare the control group (CON) with the SHAM group.

PRE-PN, value obtained before clamping the pulmonary artery; PRE-RP, value obtained before starting the reperfusion; 30'P-RP and 30'P-RP, values obtained 30 min and 60 min after reperfusion respectively.

*p<0.01 vs. all; **p<0.001 vs. PRE-PN and PRE-RP

Figures.

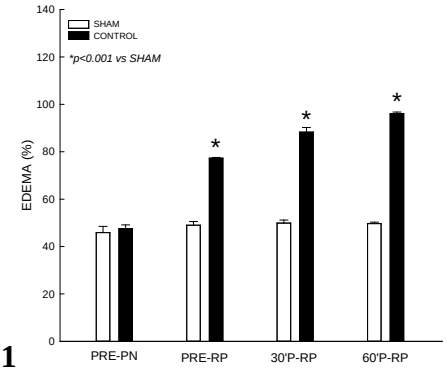


Figure 1

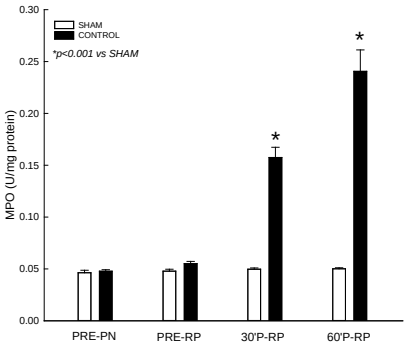
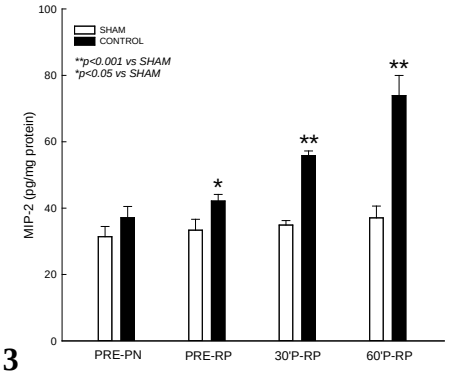


Figure 2



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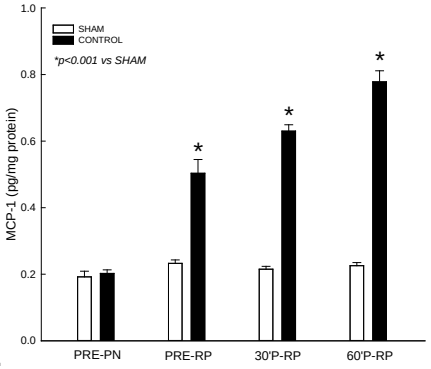

Figure 4

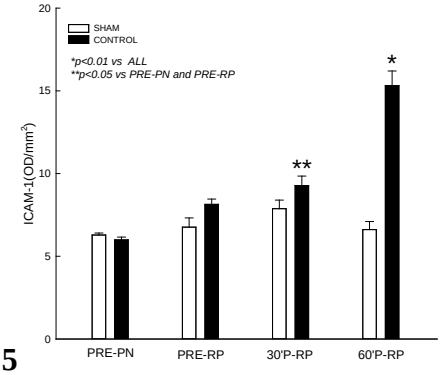

Figure 5

ARTÍCULO VII

Systemic and alveolar inflammatory response in the dependent and nondependent lung in patients undergoing lung resection surgery.

Francisco de la Gala, Patricia Piñeiro, Ignacio Garutti, Almudena Reyes, Luis Olmedilla, Patricia Cruz, Patricia Duque, Javier Casanova, Lisa Rancan, Pilar Benito and Elena Vara

ORIGINAL ARTICLE

Systemic and alveolar inflammatory response in the dependent and nondependent lung in patients undergoing lung resection surgery

A prospective observational study

Francisco de la Gala, Patricia Piñeiro, Ignacio Garutti, Almudena Reyes, Luis Olmedilla, Patricia Cruz, Patricia Duque, Javier Casanova, Lisa Rancan, Pilar Benito and Elena Vara

BACKGROUND Measurement of inflammatory mediators in bronchoalveolar lavage (BAL) during lung resection surgery with periods of one-lung ventilation (OLV) has revealed an intense local pulmonary response. The role of each lung in the inflammation that occurs during this procedure has never been investigated.

OBJECTIVE(S) The primary objective of our study was to compare the inflammatory response in the dependent lung with that of the nondependent lung by measuring inflammatory markers in BAL. Our secondary objective was to assess the behaviour of these inflammatory mediators in patients with and without postoperative pulmonary complications (PPCs).

DESIGN A prospective, observational study.

SETTING Department of Anaesthesiology in a university hospital.

PATIENTS Forty-six consecutive patients undergoing lung resection surgery.

INTERVENTION(S) BAL samples were taken from dependent and nondependent lung 5 min before initiating OLV and at the end of OLV (once two-lung ventilation was established). All patients were followed up until 30 days after surgery.

MAIN OUTCOME MEASURES The concentration of cytokines [interleukin (IL)-1, IL-2, IL-6, IL-10, tumour necrosis factor-alpha (TNF-α)], nitric oxide, carbon monoxide and matrix metalloproteinase 2 (MMP-2) was analysed in both lungs before and after OLV. PPCs were recorded.

RESULTS In BAL fluid, all measured biomarkers, apart from IL-10, were significantly greater ($P < 0.05$) at the end of OLV than those obtained before OLV, both for the dependent and nondependent lung. The increase in measured biomarkers was similar in both lungs. Eight patients developed PPC. Patients who developed PPC had higher levels of TNF-α ($P < 0.05$) in BAL from the nondependent lung before and after OLV than patients who did not have PPC. Patients who developed PPC had a smaller increase in MMP-2 levels ($P < 0.05$) in the dependent lung than patients who did not have PPC.

CONCLUSION In lung resection surgery, the inflammatory response is similar in both lungs. However, the greater increase in TNF-α levels in the nondependent lung and the smaller increase of MMP-2 concentration in the dependent lung may increase the susceptibility to develop PPC.

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Introduction

Noncardiac thoracic surgery involves a greater systemic and pulmonary inflammatory response and is associated with more complications than major abdominal procedures.[1] Although several explanations for this phenomenon have been postulated, the underlying causative mechanisms have not been fully elucidated. The fact that major thoracic procedures (oesophagectomy and pneumonectomy) are performed using a similar surgical approach suggests that the use of intraoperative one-lung ventilation (OLV) and/or contralateral collapse lung and

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surgical manipulation may contribute to the greater inflammatory response seen in comparison to abdominal surgery.[2–6]

Measurement of specific inflammatory mediators in bronchoalveolar lavage (BAL) fluid during lung resection surgery (LRS), which requires the use of OLV, has revealed an intense local pulmonary response, for which various pathophysiological mechanisms have been postulated.[7–11] These include pulmonary oxidative stress in both lungs, injury caused by mechanical ventilation during OLV and/or ischaemia/reperfusion phenomena in the dependent lung and surgical manipulation of the nondependent lung.[12–16] However, due to the range of possible underlying mechanisms, more detailed evidence is necessary on the role of each lung in the development of the inflammation that invariably occurs during this procedure.

Most studies evaluating the inflammatory response in the lungs report findings for BAL fluid from only one lung and disregard the fact that, in the other lung, the inflammatory response may be different in terms of cause and intensity. In recent years, some authors have analysed the inflammatory response in each lung individually during surgical procedures involving the chest, lungs and oesophagus.[10,11,17,18] Findings were contradictory; some authors found no differences in the inflammatory response in each lung, while others concluded that the dependent lung was the main source of inflammatory mediator release. This observation has been associated mainly with lung injury caused by mechanical ventilation and the use of lung-protective ventilation during OLV has provided an attenuation of the inflammatory lung response.[19]

Furthermore, the proinflammatory state triggered during and after major surgery is responsible for the adverse events that arise during the postoperative period.[5,6,20] Similarly, the pulmonary or local inflammatory response has been associated with the onset of postoperative pulmonary complications (PPCs).[9,21–24]

The hypothesis tested was that the main pulmonary inflammatory response is due to the nondependent lung, when a lung-protective ventilation strategy is applied in the dependent lung.

The primary objective of our study was to compare the inflammatory response in the dependent lung with that of the nondependent lung by measuring inflammatory markers [primarily tumour necrosis factor-alpha (TNF-α)], in BAL fluid, from each lung in patients undergoing LRS with periods of OLV and a lung-protective ventilation strategy. Our secondary objective was to assess the behaviour of these inflammatory mediators in patients with and without PPC.

Materials and methods

The present prospective, single-centre study is a substudy of a phase IV clinical trial [Study of pulmonary and systemic inflammatory response secondary to pulmonary resection surgery using intravenous anaesthesia versus inhalation anaesthesia with halogenated agents (EudraCT 2011-002294-29)], which was approved by the Area 1 Clinical Investigation Ethics Committee (No. 181/11), Madrid, Spain (Chairperson Dr Fernando Diaz Otero) on 1 August 2011. The study population included 46 patients who underwent LRS and voluntarily agreed to participate in the study by giving their signed informed consent. All of the patients fulfilled the inclusion criteria [patients of either sex undergoing LRS; voluntary acceptance to participate in the study; signed informed consent; age >18 years and legally competent; scheduled surgery; forced expiratory volume in the first second (FEV_1) >50% or forced vital capacity (FVC) >50% of predicted values; no previous chronic treatment or treatment with corticosteroids or immunosuppressive agents 3 months before surgery]. The exclusion criteria were as follows: pregnancy and breastfeeding; transfusion of blood products during the previous 10 days; impossibility of performing a lung-protective ventilation strategy; and heart failure (New York Heart Association Functional Class 3 or 4) during the week before surgery. Patients were recruited consecutively.

Analgesia was administered via a paravertebral catheter in the hemithorax of the thoracotomy incision site with an initial dose of 0.5% bupivacaine (0.3 ml kg^{-1}) followed by continuous infusion thereafter (6 to 10 ml h^{-1}). Anaesthesia was induced with propofol (2 to 3 mg kg^{-1}) and fentanyl (3 μg kg^{-1}). Neuromuscular blockade was achieved with rocuronium (0.6 to 1 mg kg^{-1}). Orotracheal intubation was performed with a double-lumen tube (35 to 37 Fr in women and 39 to 41 Fr in men). Correct placement was verified by direct visualisation with a fibreoptic bronchoscope. All patients were managed with using a Primus ventilator (Drägerwerk AG & Co. KGaA, Lübeck, Germany). Volume-controlled ventilation was used during two-lung ventilation (TLV) with the following parameters: tidal volume 8 ml kg^{-1} (ideal body weight); positive end-expiratory pressure (PEEP) 3 to 5 cmH_2O; fraction of inspired oxygen (FiO_2) 0.4 to 0.5; and respiratory rate to maintain end-tidal carbon dioxide pressure ($ETCO_2$) at 4 to 4.7 kPa. Anaesthesia was maintained with sevoflurane at the necessary concentrations to maintain a bispectral index of between 40 and 60. OLV was undertaken using volume-controlled ventilation and the following parameters: tidal volume 6 ml kg^{-1} (ideal body weight); PEEP 5 cmH_2O; permissive hypercapnia; and FiO_2 0.6 to 1 in order to maintain oxygen saturation (SaO_2) more than 90%. Recruitment manoeuvres and the application of continuous positive airway pressure (CPAP) to the nondependent lung were instituted in the event of hypoxemia. A restrictive intravenous fluid therapy was used, with fluid given at 2 ml kg^{-1} h^{-1} in order to maintain a urine output of more than

0.5 ml kg^{-1} h^{-1}. The radial artery was catheterised in all cases (FloTrac Sensor; Edwards Lifesciences Corp., Irvine, California, USA) for intraoperative monitoring of continuous cardiac output, cardiac index, stroke volume variation, stroke volume, stroke volume index and invasive arterial pressure. These values were recorded at baseline during ventilation for both lungs, at 30 min after initiation of OLV and at the end of OLV.

The parameters recorded during surgery were as follows: baseline values for TLV 30 min after initiation of OLV and at the end of OLV; FiO_2; peripheral oxygen saturation (SpO_2); $ETCO_2$; TV; minute volume; respiratory rate; peak, plateau, mean and end-expiratory airway pressures; and lung compliance. Arterial blood was drawn for measurement of respiratory gases (paO_2, SaO_2 and $paCO_2$) and inflammatory markers [interleukin (IL)-1, IL-2, IL-6, IL-10, TNF-α, nitric oxide, carbon monoxide and matrix metalloproteinase 2 (MMP-2)] at five time points: baseline (before OLV); 30 min after initiation of OLV; at the end of OLV; 6 h after surgery; and 18 h after surgery. BAL samples were taken from both lungs (dependent and nondependent) 5 min before initiating OLV and at the end of OLV (once TLV was established). Sampling was performed using a 4.5 mm fibreoptic bronchoscope wedged into the selected segment of the bronchus of the left lower lobe and middle or right lower lobe with 100 ml 0.9% saline solution in 25-ml aliquots for analysis of pulmonary inflammatory markers (IL-1, IL-2, IL-6, IL-10, TNF-α, nitric oxide, carbon monoxide and MMP-2). Blood and BAL samples obtained for determination of inflammatory markers were filtered using sterile gauze and centrifuged at 400*g* for 15 min at 4°C. The supernatant was stored at −20°C until analysis at a specialised laboratory. Concentrations of cytokines, nitric oxide and MMP-2 were analysed using western blot; carbon monoxide was analysed using the method of Omura and Sato.[25]

PPCs that may have been correlated with the initial lung inflammatory response [atelectasis, pneumonia and acute respiratory distress syndrome (ARDS)], cardiological complications, length of stay in the ICU, length of hospital stay and mortality at 30 days were recorded. All the patients were followed up after surgery until discharge. Atelectasis was evaluated using the Wisconsin scale,[26] pneumonia was diagnosed on the basis of criteria by Garner *et al.*[27] and ARDS was defined according to the Berlin criteria.[28]

Statistical analysis

From a clinical perspective, we estimated that, when comparing lung inflammatory responses, an increase in inflammatory markers greater than 20% between dependent and nondependent lungs could be considered as relevant. TNF-α is the main cytokine used to evaluate this lung inflammatory response in BAL samples.

The sample size was calculated to assess a difference of more than 20% between the values of TNF-α in both lungs after reinitiating TLV. For a two-tailed hypothesis with alpha set at 0.2 and 80% power, the required sample size was 46 patients. Differences in concentration change between the dependent and nondependent lung at a specific sample moment were evaluated by Student's *t*-test. Student's *t*-test was used to compare the percentage changes in cytokine BAL fluid levels in both lungs. When continuous variables were not normally distributed, data were analysed using the Mann–Whitney *U*-test. To compare cytokines in BAL fluid in the same lung before and after OLV, a paired *t*-test was employed. We made repeated measurement analysis of variance. Correlation between cytokines in blood and both lungs was performed by Pearsońs coefficient. Categorical data were compared using the Chi-square test. Statistical significance was set at P value less than 0.05. The statistical analysis was performed using SPSS (version 17.0; IBM, Armonk, New York, USA)

Results

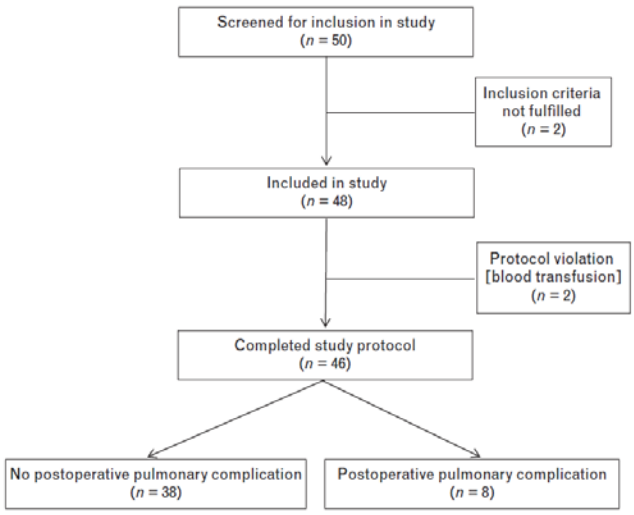
Of the 50 consecutive patients scheduled for elective LRS, 48 were included in the study. Two of the enrolled patients were excluded because of the need for a blood transfusion during the surgery. Forty-six patients successfully completed the study (Fig. 1). Patient demographic, surgical and preoperative respiratory data are summarised in Table 1. Intraoperative haemodynamic and respiratory data are summarised in Table 2.

The analysis of BAL fluid revealed that all biomarkers measured, with the exception of IL-10, were significantly greater at the end of OLV than those obtained before OLV, both for the dependent lung and the nondependent lung. The increase in all biomarkers measured in BAL fluid was similar in both lungs. MMP-2 was the biomarker that demonstrated the greatest increase, in both lungs, after OLV (Table 3).

With the exception of carbon monoxide and nitric oxide, the mean plasma cytokine levels increased during and after surgery in all patients (Table 4). During surgery, the concentration of cytokines was greater in BAL fluid than in plasma in baseline and at the end of OLV. Pearsońs coefficient of significant correlation between plasma and BAL fluid (dependent and nondependent lung) cytokines is summarised in Table 5.

PPCs were recorded in eight patients (three pneumonias, three atelectasis and two ARDS) all of whom had higher levels of TNF-α in BAL fluid from the nondependent lung before and after OLV than patients who did not have complications (Fig. 2 and Table 6). However, in the dependent lung, the levels of these markers were similar for patients who developed complications and those who did not. Patients who developed PPC had a lower increase in MMP-2 levels in the dependent lung than those who

Fig. 1



~~Tumour necrosis factor-alpha in bronchoalveolar lavage samples of both lungs in patients with or without postoperative pulmonary complication.~~

did have PPC. Furthermore, we did not observe differences between these groups in the comparison of perioperative plasma cytokine values (Table 7).

Discussion

Pulmonary inflammatory response

In concordance with other investigations, we observed an increased pulmonary inflammatory response in patients undergoing LRS with periods of OLV. Different pathophysiological mechanisms have been proposed to explain the inflammatory response in each lung. Our results, however, showed that the intensity of this exaggerated lung inflammatory response was similar in both the dependent and nondependent lung. This similar inflammatory response in both lungs suggests that no clear predominant cause has been recognised to date. Surgical manipulation and oxidative stress secondary to ischaemia-reperfusion induces an inflammatory response in the nondependent lung. Moreover, the biological effect produced not only effects this lung but also the contralateral lung and other organs.[12,29,30] In the same way, the inflammatory response in the dependent lung is basically triggered by mechanical ventilation and oxidative stress secondary to hyperoxia, although it also affects the nondependent lung.

Table 1 Surgical and demographic data

Lobectomy/segmentectomy	30/16
Duration of operation (min)	281 (86)
Duration of one-lung ventilation (min)	197 (85)
Side (right/left)	25/21
Sex (male/female)	34/12
ASA (I/II/III)	4/29/13
Age (years)	62 (12)
Weight (kg)	72 (15)
Height (cm)	169 (9)
Forced expiratory volume in the first second [FEV_1] (% predicted value)	91 (19)
Forced vital capacity [FVC] (% predicted value)	106 (19)
FEV_1/FVC ratio	73 (6)

All data are mean (SD) or number. ASA, American Society of Anesthesiologists' physical classification.

Table 2 Intraoperative haemodynamic and respiratory data

	Baseline	OLV	TLV
paO_2 (kPa)	21.86 (8.6)	10.79 (3.1)	24.79 (10.1)
FiO_2	0.48 (0.07)	0.81 (0.15)	0.59 (0.15)
paO_2/FiO_2	337 (122)	102 (31)	319 (107)
$paCO_2$ (kPa)	6.41 (0.8)	6.98 (1.2)	6.55 (1.3)
Peak airway pressure (cmH_2O)	19.8 (4.2)	24.4 (4.1)	19.9 (5.6)
Plateau airway pressure (cmH_2O)	17.3 (3.4)	19.7 (4.2)	16.5 (5.0)
Mean airway pressure (cmH_2O)	8.6 (1.3)	10.2 (1.8)	8.7 (2.0)
Lung compliance (cmH_2O)	40.1 (11.2)	29.0 (7.5)	43.9 (14.8)
Mean arterial pressure (mmHg)	82 (17)	78 (14)	82 (13)
Heart rate (bpm)	76 (14)	76 (15)	79 (13)
Cardiac index ($ml\,min^{-1}\,m^{-2}$)	2.83 (0.8)	2.88 (0.8)	3.05 (0.9)
Stroke volume variation (%)	13 (6)	10 (5)	11 (6)
Stroke volume index ($ml\,min^{-1}$)	38 (10)	40 (18)	38 (11)
Haemoglobin ($g\,dl^{-1}$)	12.8 (1.5)	12.8 (1.5)	12.4 (1.6)
BIS	46 (7)	45 (7)	46 (7)

All data are mean (SD) or number. Baseline, 10 min prior to commencing OLV; OLV 30, 30 min after the start of OLV; TLV, two-lung ventilation recorded 10 min after reinstitution of TLV. BIS, bispectral index; FiO_2, inspired fraction of oxygen; OLV, one-lung ventilation; TLV, two-lung ventilation.

Table 3 Biomarkers in bronchoalveolar lavage samples

	Nondependent lung				Dependent lung				% change dependent versus nondependent lung
	Baseline	End	P	% change	Baseline	End	P	% change	P
IL-1 (pg ml^{-1})	137 (123 to 160)	204 (180 to 233)	0.000	42 (23 to 69)	140 (120 to 151)	199 (175 to 236)	0.000	46 (29 to 78)	0.205
MMP-2 (ng ml^{-1})	3.95 (3.6 to 5.6)	8.47 (7.3 to 9.6)	0.000	91 (62 to 152)	4.12 (3.3 to 5.7)	8.31 (7.3 to 9.7)	0.000	110 (51 to 151)	0.632
NO (nmol ml^{-1})	8.32 (7 to 9.8)	8.82 (7.3 to 9.6)	0.048	26 (-20 to 28)	8.25 (6.5 to 9.7)	8.35 (6.2 to 9.8)	0.000	-7 (-24 to 25)	0.321
CO (pmol ml^{-1})	6.8 (5.7 to 7.9)	7 (5.8 to 8.3)	0.000	4 (-3 to 16)	6.44 (5.9 to 7.7)	6.6 (5.7 to 8.1)	0.000	0 (-9 to 13)	0.276
IL-10 (pg ml^{-1})	41.7 (40 to 46)	42.3 (40 to 45)	0.605	-11 (-8 to 8)	41 (39.7 to 44.3)	41.9 (39.2 to 46)	0.559	0 (-7 to 13)	0.363
IL-2 (pg ml^{-1})	2.36 (1.96 to 2.8)	2.44 (2.02 to 2.83)	0.000	-12 (-14 to 10)	2.31 (1.98 to 2.94)	2.52 (1.94 to 2.94)	0.000	2 (-15 to 6)	0.971
IL-6 (pg ml^{-1})	6.85 (5.3 to 7.8)	7.01 (5.8 to 8)	0.000	56 (-14 to 32)	608 (4.9 to 7.3)	6.3 (5.3 to 7.5)	0.000	4 (-7 to 18)	0.891
TNF-α (pg ml^{-1})	17.1 (14.5 to 21.4)	17.8 (14.6 to 22)	0.000	18 (-7 to 14)	16.5 (12.2 to 21.6)	16.9 (14.1 to 23.3)	0.000	6 (-3 to 12)	0.602

All data are median (IQR). Baseline, 10 min prior to commencing one-lung ventilation; End, completion of surgery prior to tracheal extubation; CO, carbon monoxide; IL, interleukin; NO, nitric oxide; TNF-α, tumour necrosis factor-alpha.

Several authors have assessed the inflammatory response during OLV in thoracic surgery.[7,8,10,11,17,31] Nevertheless, few studies have investigated the inflammatory response of both lungs in the same patient during this procedure.[10,11,18,19] These studies have reported contradictory results. Sugasawa *et al.*[10] studied 20 patients and found a more pronounced pulmonary inflammatory response at the end of surgery (as seen in elevated levels of IL-1, IL-6 and IL-8) in the dependent lung than the nondependent lung. In a subsequent study (40 patients), the same group found a different result, with significantly increased concentrations (compared with baseline) of the same cytokines both in the dependent and nondependent lung at the end of OLV.[11] The authors concluded that this inflammatory response can be induced by several factors, including stress caused by surgical manipulation, lung collapse and re-expansion caused by OLV, damage caused by using high inspiratory concentrations of oxygen and the damage caused by high inspiratory pressure during OLV. Our results support the conclusions of the authors.

In contrast, Zingg *et al.*[18] studied 15 patients undergoing LRS and observed a more pronounced inflammatory response in the nondependent lung than in the dependent lung. The same authors had previously analysed patients undergoing transthoracic oesophagectomy with OLV, in whom they observed a more pronounced reaction in the dependent lung.[19] This difference could be explained by the fact that LRS causes damage directly to the lung tissue, which in turn triggers a more severe reaction on the operated side that is subsequently measured in the BAL fluid. However, in oesophagectomy, wherein the nondependent lung is not directly manipulated, the greater part of the inflammatory response could be due to ventilation of the dependent lung during OLV via high concentrations of oxygen and the lung damage caused by direct mechanical stress in the alveolar walls.[19]

Our findings are in contrast with the results cited above, except for those of Sugasawa *et al.*,[11] although our sample was larger and we observed no differences in the inflammatory response of each lung.

TNF-α is considered the main biomarker to evaluate lung inflammation. It is mainly produced by macrophages and monocytes, with increased concentrations in BAL fluid reflecting an increase in the quantity of alveolar macrophages. Clinical and experimental studies of the impact of mechanical ventilation on pulmonary immune function revealed progressive alteration of this function, which is characterised by aggregation of alveolar neutrophils and macrophages.[32,33] In their clinical studies, Schilling *et al.*[7,8] found increased intra-alveolar granulocytes and TNF-α in the dependent lung of patients undergoing thoracic surgery with OLV, but these studies did not measure TNF-α levels in the nondependent

Table 4 Intraoperative and postoperative plasma biomarkers

	Baseline	OLV 30	End	6 h postop	18 h postop
IL-1 (pg ml^{-1})	27.6 (10)	31.7 (11)***	31.8 (10)***	33.4 (17)**	31.3 (11)**
TNF-α (pg ml^{-1})	6.9 (2)	8.3 (3)***	9.3 (3)***	9.1 (3)***	8.1 (2)***
IL-6 (pg ml^{-1})	3.1 (0.6)	3.5 (1)*	4.1 (1.2)***	3.6 (1.3)*	3.5 (0.9)**
IL-2 (pg ml^{-1})	0.9 (0.1)	1.26 (0.2)***	1.3 (0.3)***	1.1 (0.3)***	1.1 (0.3)***
CO (pmol ml^{-1})	2.62 (0.3)	2.74 (0.3)	2.81 (0.3)*	2.8 (0.2)*	2.9 (0.2)*
IL-10 (pg ml^{-1})	0.09 (0.01)	0.11 (0.01)***	0.10 (0.01)***	0.09 (0.01)**	0.09 (0.01)***
NO (nmol ml^{-1})	29.6 (11)	28.7 (12)	34.1 (12)*	25.8 (7)*	27.3 (10)

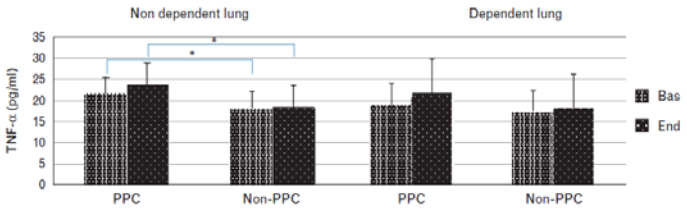
All data are mean (SD). Baseline, 10 min prior to commencing one lung ventilation (OLV); OLV 30, 30 min after starting OLV; End, surgery complete but prior to tracheal extubation; Postop, postoperative. CO, carbon monoxide; IL, interleukin; NO, nitric oxide; TNF-α, tumour necrosis factor-alpha. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus baseline.

Table 5 Correlation between cytokines measured in plasma and bronchoalveolar lavage fluid of both lungs

			Plasma cytokines									
			Dependent lung					Nondependent lung				
			Baseline	OLV 30	End	6 h Postop	18 h Postop	Baseline	OLV 30	End	6 h Postop	18 h Postop
Bronchoalveolar lavage fluid cytokines	IL-2 (Baseline)	*r*	0.103	0.306	0.11	0.091	0.071	0.09	0.358*	0.123	0.460	0.416
		P	NS	0.046	NS	NS	NS	NS	0.018	NS	0.002	0.006
	IL-2 (End)	*r*	0.087	-0.019	-0.024	-0.051	-0.056	0.11	0.029	0.081	0.400	-0.001
		P	NS	NS	NS	NS	NS	NS	NS	NS	0.009	NS
	IL-1 (Baseline)	*r*	-0.1	0.029	0.114	0.016	0.073	-0.034	-0.062	-0.02	-0.024	-0.015
		P	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	IL-1 (End)	*r*	0.135	0.071	0.350	0.406	0.114	0.093	0.123	0.114	0.426	0.452
		P	NS	NS	0.027	0.009	NS	NS	NS	NS	0.004	0.003
	NO (Baseline)	*r*	-0.350	-0.343	0.025	0.052	-0.048	-0.337	0.126	-0.001	0.068	0.01
		P	0.020	0.025	NS	NS	NS	0.025	NS	NS	NS	NS
	NO (End)	*r*	0.119	0.126	0.071	0.098	0.121	-0.398	-0.403	0.043	-0.02	-0.372
		P	NS	NS	NS	NS	NS	0.008	0.008	NS	NS	0.018

Baseline, 10 min before commencement of OLV; End, surgery complete but prior to tracheal extubation; NO, nitric oxide; NS, not significant; OLV 30, 30 min after the start of one-lung ventilation (OLV); Postop, postoperative; *r*, Pearson correlation coefficient..

Fig. 2



PPC Pulmonary postoperative complications. Bas: before one-lung ventilation
End: after reinitiating two-lung ventilation
(*): $p < 0.05$ PPC vs non PPC

tumor necrosis factor alpha in bronchoalveolar lavage samples of both lungs in patients with or without postoperative pulmonary complication. AQ3

lung. De Conno *et al.*[9] studied the inflammatory response in BAL fluid from the nondependent lung in patients scheduled for LRS with periods of OLV who were randomised in two groups depending on the anaesthetic agent employed (sevoflurane or propofol). They found higher TNF-α values at the end of the surgery in both groups, with smaller increases in the sevoflurane group. Therefore, our finding that TNF-α increases after OLV in the dependent and nondependent lung seems justified.

Recent studies have shown an immunomodulatory effect of sevoflurane in the pulmonary inflammatory response, which is not observed with propofol. Our group in an experimental in-vivo study showed that sevoflurane exerts an early cell-protective effect against ischaemia/

Table 6 Postoperative pulmonary complications and bronchoalveolar lavage biomarkers

			Nondependent lung			Dependent lung		
	PPC	*n*	Baseline	End	% change (baseline versus end)	Baseline	End	% change (baseline versus end)
IL-1 (pg ml^{-1})	YES	8	148 (22)	215 (36)	46.5 (32)	142 (24)	188 (40)	34.9 (37)
	NO	38	138 (25)	208 (34)	47 (44)	135 (31)	207 (37)	59 (32)
MMP-2 (pg ml^{-1})	YES	8	4.63 (1)	7.25 (2)	72.9 (76)	5.27 (1)	8.36 (1)	67.8 (52)
	NO	38	4.64 (2)	8.74 (2)	108.3 (61)	4.27 (1)	8.87 (2)	127.5 (78)*
NO (nmol ml^{-1})	YES	8	8.18 (1)	8.17 (1)	2.1 (24)	9.6 (3)	7.92 (2)	-8.1 (32)
	NO	38	8.49 (3)	8.71 (3)	11.4 (52)	8.38 (3)	8.08 (3)	3.0 (43)
CO (pmol ml^{-1})	YES	8	6.89 (1)	6.94 (2)	0.3 (10)	6.11 (1)	5.78 (1)	-5 (18)
	NO	38	6.83 (1)	7.18 (1)	8.3 (19)	6.85 (1)	7.09 (1)	4.9 (21)
IL-10 (pg ml^{-1})	YES	8	42.5 (3)	40.3 (4)	-5.2 (11)	40.9 (1)	44.8 (5)	9.4 (16)
	NO	38	43.7 (5)	43.2 (5)	0.5 (12)	42.7 (6)	42 (6)	0.1 (18)
IL-2 (pg ml^{-1})	YES	8	2.79 (1)	2.67 (1)	-2.3 (13)	2.14 (1)	2.34 (1)	10.9 (13)
	NO	38	2.46 (1)	2.36 (1)	-1.6 (17)	2.55 (1)	2.49 (1)	-2.2 (20)
IL-6 (pg ml^{-1})	YES	8	6.9 (1)	7.55 (1)	9.8 (14)	5.71 (1)	6.99 (1)	24.3 (34)
	NO	38	6.69 (2)	6.88 (1)	8.4 (24)	6.2 (2)	6.48 (2)	6.2 (22)
TNF-α (pg ml^{-1})	YES	8	21.5 (4)	23.6 (4)	11.1 (20)	18.9 (8)	21.7 (9)	15.3 (13)
	NO	38	18.1 (5)*	18.4 (6)*	3.1 (18)	17.3 (5)	18.2 (5)	5.2 (18)

Patients with postoperative pulmonary complications versus those without. Baseline, 10 min prior to commencing one-lung ventilation; CO, carbon monoxide; End, completion of surgery prior to tracheal extubation; IL, interleukin; NO, nitric oxide; PPC, postoperative pulmonary complications. *$P < 0.05$.

Table 7 Postoperative pulmonary complications and plasma cytokines

	PPC	*n*	Baseline	OLV 30	End	6 h Postop	18 h Postop
IL-1 (pg ml^{-1})	Yes	8	29.3 (16.1)	34.2 (16.4)	29.1 (6.7)	37.8 (23.5)	34.9 (15.3)
	No	38	27.2 (6.8)	31.0 (9.0)	32.8 (10.3)	32.5 (14.8)	30.2 (8.4)
TNF-α (pg ml^{-1})	Yes	8	6.88 (0.8)	7.86 (1.7)	9.58 (3.0)	9.53 (4.3)	7.91 (1.4)
	No	38	6.94 (1.9)	8.43 (2.9)	9.42 (3.2)	9.03 (3.6)	8.15 (1.7)
IL-6 (pg ml^{-1})	Yes	8	3.07 (0.6)	3.49 (0.5)	4.05 (1.5)	3.90 (1.1)	3.41 (0.7)
	No	38	3.13 (0.6)	3.57 (1.1)	4.14 (1.2)	3.51 (1.3)	3.55 (1.0)
IL-2 (pg ml^{-1})	Yes	8	0.85 (0.1)	1.24 (0.2)	1.28 (0.3)	1.07 (0.3)	1.17 (0.4)
	No	38	0.88 (0.1)	1.27 (0.3)	1.27 (0.4)	1.1 (0.3)	1.08 (0.2)
CO (pmol ml^{-1})	Yes	8	2.60 (0.4)	2.74 (0.3)	2.84 (0.4)	2.91 (0.2)	2.90 (0.3)
	No	38	2.64 (0.5)	2.75 (0.3)	2.8 (0.3)	2.78 (0.2)	2.86 (0.2)
IL-10 (pg ml^{-1})	Yes	8	0.09 (0.0)	0.10 (0.0)	0.11 (0.0)	0.09 (0.0)	0.10 (0.0)
	No	38	0.09 (0.0)	0.11 (0.0)	0.10 (0.0)	0.09 (0.0)	0.09 (0.0)
NO (nmol ml^{-1})	Yes	8	29.5 (14.3)	31.8 (13.3)	41.6 (15.7)	29.2 (5.2)	25.5 (7.3)
	No	38	29.6 (9.7)	28.2 (11.7)	32.0 (11.0)	24.7 (7.5)	28.3 (10.5)

All data are mean (SD). Baseline, 10 min prior to commencing one lung ventilation (OLV); CO, carbon monoxide; End, surgery complete but prior to tracheal extubation; IL, interleukin; NO, nitric oxide; OLV 30, 30 min after starting OLV; Postop, postoperative; PPC, postoperative pulmonary complications.

reperfusion pulmonary damage, as evidenced by a decrease in oxidative stress and in the intensity of the inflammatory response.[8,9,34]

Systemic inflammatory response

The significant increase in the plasma cytokines analysed proves the existence of a systemic inflammatory response in patients undergoing LRS. Noncardiac thoracic procedures (LRS or oesophagectomy performed by thoracotomy with OLV) seem to trigger a more pronounced systemic inflammatory response than major abdominal procedures.[1] This is probably because the damage caused by thoracotomy with periods of OLV is a stress factor, which is independent of the duration of the surgery and anaesthesia and blood transfusion.[1] This hypothesis is supported by the results of several studies that show lower systemic expression of pro and anti-inflammatory cytokines in video-assisted thoracic surgery than in open thoracic surgery.[2,35] Some authors have postulated that activation of cellular immunity in the lung may be partially responsible for the increased systemic inflammatory response, owing to the decompartmentalisation of cytokines as they pass from the lung to the bloodstream.[23] However, in our investigation, we did not find a clear relationship between systemic and pulmonary inflammatory response. We believe that the use of lung-protective ventilation with low tidal volumes during OLV in our study was the probable reason. Previous studies have shown that the attenuation of the pulmonary inflammatory response associated with lung-protective ventilation during OLV in patients undergoing LRS did not change the plasma concentrations of TNF-α, IL-6, IL-10 and IL-1.[17,36,37] Michelet *et al.*[23] studied patients undergoing more aggressive interventions (oesophagectomy) and found that lung-protective ventilation was associated with reduced plasma concentrations of IL-8, IL-6 and

IL-1. Therefore, the systemic inflammatory response seems to depend on the invasiveness of the surgical procedure (oesophagectomy >LRS by thoracotomy >LRS by thoracoscopy).[38]

Postoperative pulmonary complications

Given that systemic and pulmonary inflammation occurs during and after LRS, there is growing evidence suggesting that high inflammatory cytokine levels may be clinically relevant in postsurgical pulmonary complications.[9,21–24] Considerable emphasis has been placed on detecting an early marker of pulmonary complications that would enable us to take therapeutic measures aimed at improving prognosis.

To our knowledge, all previous studies that have tried to associate levels of pulmonary inflammatory mediators with postoperative course did not take into account variations in the concentration of these mediators in each lung. We found that percentage changes of pulmonary mediators were similar in each lung of patients with PPC.

We did not find an association between cytokine levels in BAL fluid from the dependent lung and onset of PPC. However, we observed that patients who developed PPC had higher levels of TNF-α in BAL fluid in the nondependent lung prior to lung collapse for OLV. A potential explanation could be that the trigger mechanisms are more associated with the baseline situation of the operative lung than with the intraoperative pulmonary management. All the patients included in the study had cancer in the nondependent lung; it is well known that tumour cells secrete cytokines and that the alveolar macrophages of patients with cancer secrete more proinflammatory cytokines than patients who do not have cancer.[39,40]

In the last years, some studies have shown the important role of MMP in ventilator-induced lung injury (VILI). Inflammation, matrix remodelling and cell migration act in an orchestrated fashion to repair the injured lung.[41,42] MMP-2 promotes epithelial cell migration and it has been suggested that it contributes to alveolar repair after VILI or ARDS. It has been observed that a greater response to mechanical ventilation in the levels of MMP-2 taken in the BAL fluid of patients with ARDS improved survival.[43] Similarly, we have seen that the attenuation of the response in the MMP-2 in the dependent lung during OLV is associated with worse postoperative course. In our knowledge, this is the first clinical investigation showing the important role of MMP-2 in PPC in the patients undergo LRS.

The sample size in our study was not calculated with the aim of detecting differences in PPC depending on the levels of inflammatory mediators measured. Therefore, our results should be interpreted with caution. Moreover, the observational nature of the study can affect the degree of evidence. However, the study was performed according to strict departmental protocols on management of anaesthesia in patients undergoing LRS.

Our study is limited by the short postsurgical observation period, the low number of patients included, the long duration of OLV in our institution and the fact that pulmonary complications are usually analysed as a secondary variable.[9,44] Duration of OLV is associated with an increased prevalence of cardiovascular and pulmonary complications.[12] There are also several limitations inherent to the techniques we used. Extraction of BAL fluid samples in both lungs is complex, slow and risky and it can alter the integrity of the distal airway. Therefore, we cannot exclude the possibility that the release of cytokines is affected by manipulation of the distal airway, especially as a result of the insertion of the double-lumen tube and extraction of BAL fluid. In addition, interpretation of alveolar inflammatory mediators in BAL fluid is problematic and open to question, as an unknown fraction remain within the cell. The analysis of BAL fluid from both lungs removes the bias attributable to the substantial variation in cytokine levels depending on each patient's condition.[9,19,44–49]

In LRS, each lung makes a similar contribution to the inflammatory response, which is much more pronounced than the systemic response. However, it could be that the increased presence of TNF-α in nondependent lung and the smaller increase in MMP-2 concentration after surgery in the dependent lung increase the susceptibility to develop PPC after LRS.

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Conflicts of interest: none.

Presentation: preliminary data were presented at Euroanaesthesia 2013 (Barcelona, Spain); accepted abstract number ESAAP2-6, under which it was published in the *European Journal of Anaesthesiology* (Volume 30, Supplement 51, June 2013).

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Congresos como autor principal

1. **L. Rancan**, L. Huerta, G. Cusatti, I. Erquicia, J. Isea, V. Garcia, I. Garutti, C. Simón, E. Vara. Sevoflurane prevents liver inflammatory response induced by lung ischaemia/reperfusion in a lung autotransplant model. 47th Annual Congress of European Society for Surgical Research. Lille, Junio 2012 **WALTER BRENDEL AWARD**

ESSR 2012 Abstract Submission

Cardiovascular and Thoracic Surgery

ESSR12-1025

SEVOFLURANE PREVENTS LIVER INFLAMMATORY RESPONSE INDUCED BY LUNG ISCHAEMIA/REPERFUSION IN A LUNG AUTOTRANSPLANT MODEL

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Preferred Presentation Method: Oral or Poster
Are you under 35 years old ?: Yes
Please enter your birth date: 02.07.1985

Introduction: Ischemia reperfusion injury (I/R) is an increasingly important clinical problem. It is believed that I/R injury induces inflammatory response, which elicits tissue damage in a number of organs. In fact, various investigators have reported that any injury to tissues causes distant-organ injury. Liver injury is one of the distant-organ damages induced by tissue I/R.
Objectives: The present study was designed to investigate a possible protective effect of sevoflurane against liver inflammatory response to lung I/R.
Materiel/Patients and Methods: Two groups (sevoflurane and control) of 10 large-white pigs were submitted to a left lung auto-transplant. Both groups received the same anesthetic induction (fentanyl 3 ug/Kg, propofol 3 mg/Kg, atracurium 0,5 mg/Kg). Hypnotic maintenance was performed with sevoflurane 4% (sevo group) or propofol 10 mg/Kg/h (control group) until pneumonectomy was done; then propofol 10 mg/Kg/h was used for the two groups. Blood and tissue samples were taken in four different moments, 1) pre-neumonectomy (5 min before pulmonary artery clamp, PreNeu), 2) pre-reperfusion (5 min before reperfusion, PR), 3)10 minutes post reperfusion (PR10) and 4) 30 min after reperfusion (PR30), in order to measure levels of different of inflammatory, oxidative stress and apoptotic biomarkers (IL1, IL-10, TNFa, MCP-1, NOS, caspase 3). Non-parametric test was used to find statistical meaning.
Results: Lung ischaemia/reperfusion (I/R) markedly increased the expression of TNF ($p<0,01$) and IL-1 ($p<0.05$) and caspase activity ($p<0.05$) in control livers compared with PreNeu levels, whereas liver IL-10 expression only was changed after 30 min post reperfusion ($p<0.05$). Sevoflurane significantly decreased lung I/R-induced, TNFα($p<0,01$) and IL-1 ($p<0,01$) liver expression and caspase 3 activity ($p<0.05$) compared with the observed in the control group. Sevoflurane also reverted the lung I/R-induced decrease on IL-10 expression ($p<0.05$). No changes were found for iNOS expression.
Conclusion: In conclusion, the present results indicate that lung IR cause distant-organ injury such as in the liver. Sevoflurane treatment attenuates lung IR-induced liver injury in a model of lung autotransplant in pigs

Disclosure of Interest: None Declared

Keywords: Liver inflammatory response, Lung ischemia/ reperfusion, Sevofluorane

2. **L. Rancan**, L. Huerta, G. Cusatti, I. Erquicia, J. Isea, V. Garcia, I. Garutti, C. Simón, E. Vara. Sevoflurane prevents liver inflammatory response secondary to lung Ischemia/Reperfusion. SARS Meeting, London, UK.Enero 2013 **INVITED SPEECH**.
3. **L. Rancan**, L. Huerta, G. Cusatti, I. Erquicia, J. Isea, V. Garcia, I. Garutti, C. Simón, E. Vara. Sevoflurane prevents liver inflammatory response in a lung autotransplant model. 8th Annual Academic Surgical Congress. New Orleans, USA, Febrero 2013. **INVITED SPEECH**.

ESSR EUROPEAN SOCIETY FOR SURGICAL RESEARCH
Newsletter 1/2013

Note from the President

Dear ESSR Members and Colleagues

It is just a few weeks before the most important event of our calendar, the Annual Congress. We are all looking forward to a great meeting in the historic yet modern and vibrant city of Istanbul. We are anticipating a record attendance. As every year I would like to warmly invite all of you, your co-workers, residents and medical students to attend the Istanbul Congress.

Sister Societies

I had the privilege of representing the ESSR at the UK Society of Academic and Research Surgeons (SARS) Annual Meeting held at the Royal Society of Medicine in London on the 9th – 10th of January 2013. The meeting was characterised by a very high quality of papers and also satellite meetings of the British Association of Urological Surgeons and the Plastic Surgery British Burns Association. The calibre of the Patey Prize session presentations was amazing.

I was also able to attend the American Society of University Surgeons (SUS – 8th Annual Academic Surgical Congress) in New Orleans in February (5th - 7th). The Congress was extremely well attended with over 1000 registered participants. It covered a very wide variety of research subjects and key note lectures on current issues facing academic surgeons.

I am pleased to report that the 2012 (Lille) ESSR Walter Brendel Prize winner, Dr. Lisa Rancan (Madrid, Spain), presented her award winning study very well during a plenary session of the SARS meeting in London and a satellite session of the SUS in New Orleans. It was entitled *'Sevoflurane prevents liver inflammatory response induced by lung ischemia/reperfusion in a lung autotransplant model'*

I extended the ESSR invitation in person to the newly elected President of the Society of University Surgeons, Dr. Joe Hines and the President-Elect of SARS, Prof Cliff Shearman, who both kindly accepted the invitations. We look forward to hosting the Presidents of the UK SARS (Prof Shearman of Southampton) and the Surgical Research Society of Southern Africa (Prof Jan H. Becker of Pretoria University) and the SUS President (Prof Joe Hines). As per tradition the SRS-SA, SUS and SARS prize winners will be presenting their papers in our prestigious Walter Brendel Session on Thursday 30th May.

4. **L. Rancan,** C.A. Calvo, D. Rincon, C. Ginés, R. Esteras, C. Simón, I. Garutti, E.Vara, Lidocaine prevents lung inflammatory response to one lung ventilation. 48th Congress of the European Society for Surgical Research (ESSR). Mayo 2013, Istanbul, Turkey

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OP 184

Lidocaine Prevents Lung Inflammatory Response To One Lung Ventilation

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Introduction: Lung resection surgery with one lung ventilation (OLV) is generally associated with an intense local and systemic inflammatory response. Lidocaine is a commonly used local anesthetic agent which has also been found to possess anti-inflammatory activity.

Objectives: This study was designed to investigate the possible effect of lidocaine on lung injury secondary to OLV.

Material/Patients and Methods: Eighteen swines undergoing left caudal lobectomy with OLV were randomly divided in 3 groups: animals receiving anesthesia with IV propofol alone (CON) or plus lidocaine (LIDO) and sham group (SHAM). Samples of bronchoalveolar lavage (BAL) and blood were collected before and after OLV and the next day. Lung biopsies from collapsed (LCL) and ventilated (ML) lungs were collected before surgery and 24h after it. Levels of inflammatory (IL-1, IL-2, TNFα, NFkB, MCP-1) and apoptotic (caspases, BAD, BAX, BAK, Bcl2) biomarkers were determinate in lung samples. BAL and blood levels of metalloproteinases (MMP) were also determined.

Results: OLV increased the expression of TNFα, IL-1, IL-2, MCP-1 and NFkB ($p<0.05$) in the LCL. the OLV effect was reduced by lidocaine. Caspase (3 and 9), BAD and BAX activities as BAL and blood MMP2 and MMP9 levels were higher ($p<0,05$) in both CON and LIDO groups compared to SHAM group ($p<0,05$) and again these effects were partially blocked by lidocaine. Lidocaine administration was associated with high values of Bcl-2 compared with the other two groups. No change was observed in BAL levels of MMP3

Conclusion: These results suggest that lidocaine prevents OLV-induced lung injury through the reduction of proinflammatory cytokines and lung apoptosis.

OP 186

Does Diagnostic Excisional Biopsy Prevent the Breast Conserving Surgery?

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Introduction: Excisional biopsy in the diagnosis of breast cancer has decreased in recent years. in experienced centers, preoperative tru-cut or FNAB are 75-80% breast cancer diagnosis. Performing excisional biopsy for diagnosis makes breast preservation surgery which will be carried out later on complicated and defaces breast cosmetically.

Objectives: in this study; we aimed to determine whether diagnosing breast cancer with excisional biopsy is defined on gynecomastia treatment, not-well established systematic approaches in technique selection have negative impacts on the treatment process and may lead to repeated surgeries and patient dissatisfaction.

Objectives: in this study, our approach to gynecomastia patients, our algorithm for selection of treatment modality and our experience are presented.

Material/Patients and Methods: in the study 32 cases of gynecomastia, who were treated in our clinic between 2010-2012 were included. According to evaluation of the patient records and photos, the parameters of volume, ptosis, width of the areola, breast content (glandular-fat) were revealed. This data were compared with the surgical technique and our algorithm for treatment selection was established.

Results: the average age of the patients were 19 (17-22). 3 of 32 patients were suffered from one-side gynecomastia. 12 patients were underwent conventional liposuction. 14 patients were underwent combination of liposuction and excision. 6 patients were applied to excision only. Skin reduction via periareolar resection were applied to 10 patients and areola reduction were applied to 6 cases.

Conclusion: Liposuction and resection, two main treatment versality. First of all, one must decide which of them is the major. Many of the literature advocate size, as a major variable in the decision-making process and many treatment algorithms had been shown in this way. As a result of this study, the content of the breast should be considered as the most important factor that determines the technique. the size and skin elasticity help us to decide skin reduction.

5. **L Rancan**, C Muñoz, C Garcìa, E. Vidaurre, I Garutti, C Simón, E Vara. Lidocaine prevents lung proinflammatory response secondary to ischemia reperfusion. XXXVII Congress of the International Union of Physiological Sciences (IUPS). Birmingham, UK. 21-26 Julio, 2013

CONTROL ID: 1682814

CURRENT THEME OR SYMPOSIUM: Cardiac & Respiratory Physiology

Topic GC: Respiratory Physiology

PRESENTATION TYPE: General Communication

TITLE: Lidocaine prevents lung proinflammatory response secondary to ischemia reperfusion

AUTHORS (FIRST NAME, LAST NAME): Lisa Rancan[1], Celia Muñoz[1], Cruz Garcìa[1], Eduardo Vidaurre[2], Ignacio Garutti[3], Carlos Simon[2], Elena Vara[1]

INSTITUTIONS (ALL): 1. Biochemistry and Molecular Biology III, School of Medicine, Complutense University, Madrid, Spain.
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3. Anesthesiology, Universitary Hospital Gregorio Maranon, Madrid, Spain.

ABSTRACT BODY: Ischemia reperfusion injury (IRI) is an increasingly important problem in clinical transplantation. Its pathogenesis involves a variety of mechanisms including the increase of proinflammatory mediators. Different prophylactic and therapeutic measures have been investigated to prevent lung injury secondary to IRI. Lidocaine (Lido) is a commonly used local anesthetic agent which has also been found to possess anti-inflammatory activity in several disorders but data available are not enough to demonstrate this fact on lung tissue. This study was designed to investigate a possible protective effect of lidocaine on lung injury secondary to IRI. Two groups (LIDO and control) of six large-white pigs each were submitted to a left lung auto-transplant. Both groups received the same anesthetic induction (fentanyl 3 ug/Kg, propofol 3 mg/Kg, atracurium 0,5 mg/Kg). In addition animals of LIDO group received a continuous IV of lidocaine 1,5 mg/kg during surgery. Lung tissue samples were taken in four different moments: 1) pre-pneumonectomy (5 min before pulmonary artery clamp), 2) pre-reperfusion (5 min before reperfusion), 3) 30 min post-reperfusion (PR30) and 4) 60 min post-reperfusion (PR60). mRNA and protein expression of different proinflammatory mediators (interleukin 1 (IL-1), tumor necrosis factor alpha (TNFa), monocyte chemoattractant protein 1 (MCP-1), interleukin 10 (IL10), and endothelial nitric oxide synthase (eNOS)) were measured. Each lung sample was immediately frozen in liquid nitrogen. mRNA expression was measured by means of RT-PCR using the SYBR Green PCR Master Mix and 300 nM concentrations of specific primers. Relative changes in gene expression were calculated using the 2-ddCt method. Western blots were used to measure the protein expression (4 replicates). In addition, plasma NO and CO levels were determined by spectrophotomety. Non-parametric tests were used to find statistical meaning. The Mann-Whitney U-test was applied to establish differences between the analysed groups. In addition, the Wilcoxon test for paired data was used to study the evolution of the intra-group values. Lung ischemia reperfusion (I/R) significantly increased both mRNA and protein expression of TNFa ($p<0.01$), IL-1 ($p<0.05$) and MCP1 ($p<0.05$). This expression was even higher after 60 minutes of reperfusion ($p<0.05$). On the contrary, I/R decreased IL10 expression ($p<0.05$). Lung I/R decreased eNOS expression ($p<0.05$) and this effect was accompanied by a decrease in plasma NO levels ($p<0,01$). These effects were blocked by lidocaine. No changes were observed in CO levels. These results suggest that lidocaine prevents I/R-induced lung injury through the reduction of proinflammatory cytokines. The administration of lidocaine might be a prospective management for preventing lung injury secondary to I/R.

6. **L Rancan**, S.D. Paredes, C Garcia, G Sánchez Pedrosa, G Cusati, Io Garutti, C Simón, E Vara. Lidocaine preserves the endothelial glycocalyx against ischaemia/reperfusion in a lung autotransplant model. 49th Congress of the European Society for Surgical Research. 21-24 Mayo 2014, Budapest, Hungary

OP170

Lidocaine Preserves The Endothelial Glycocalyx In A Lung Transplant Model

ABSTRACT DETAILS

Presentation Method	Oral
Keywords:	Lidocaine glycocalyx lung autotransplant
Category	Thoracic Surgery
Background	Healthy vascular endothelium is coated by the glycocalyx, important in endothelial functions, but destroyed by ischaemia/reperfusion (/IR). Lidocaine is a local anesthetic which has antiinflammatory activity. The influence of lidocaine on glycocalyx structure has not been investigated. This study aimed to investigate a possible protective effect of lidocaine on lung glycocalix injury secondary to I/R injury.
Material and Methods	Two groups (lidocaine and control) of 6 pigs each were submitted to a left lung autotransplant. All groups received the same anaesthetic induction. In addition animals of lidocaine group received a continuous IV administration of lidocaine during surgery. In order to measure the levels of inflammatory (ICAM-1, VCAM-1, IL-1β, TNFα), anti-inflammatory (IL10) and glycocalix degradation (syndecan-1) markers, blood and lung tissue samples were taken: 1) 5 minutes before pulmonary artery clamp (PPn), 2) 5 min before reperfusion (PRp), 3) 30 min post-reperfusion (PR30), 4) 60 min post-reperfusion (PR60).
Result	In lung samples, levels of all inflammatory markers were markedly higher ($p<0.05$) at PR30 timepoint compared to PPn and PRp values. This increase was higher at PR60. I/R decreased IL10 expression. I/R increased syndecan-1 and ICAM concentrations in plasma ($p<0.05$). Syndecan-1 levels decreased in the lung ($p<0.05$). These effects were partially blocked by lidocaine.
Conclusion	These results suggest that lidocaine prevents I/R-induced glycocalyx injury in a lung autotransplant model through decreases in proinflammatory cytokines and adhesion molecules. This strategy of lidocaine administration might be a prospective management for preventing lung injury secondary to I/R.
Presenter details	Essr Member,Under 35 Years,Trainee Under 30 Years

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7. **L Rancan**, E Marchal-Duval, S.D. Paredes, K Aymonnier, C García, C Simón, I Garutti, E Vara. MicroRNA Biomarkers for Detection of lung ischemia reperfusion injury. The FEBS EMBO 2014 Conference, Paris, France. 30 Agosto – 4 Septiembre 2014

MON-051
MicroRNA biomarkers for detection of lung ischemia reperfusion injury

L. Rancan[1], E. Marchal-Duval[1], S. D. Paredes[2], K. Aymonnier[1], M. C. Garcia[1], C. Simon[3], I. Garutti[4], E. Vara[1]
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Background: Ischemia reperfusion injury (IRI) is a leading cause of acute lung injury, a common problem in various clinical settings associated with significant morbidity and mortality. MicroRNAs (miRs) are a class of small single stranded noncoding RNAs that function as post-translational regulators of specific target mRNAs. Changes of miRs expression have been associated with different diseases and pathophysiological conditions. Accumulating evidence underlines a critical function for miRNAs in the modulation of innate and adaptive immune responses. In the last years they have emerged as regulators of IRI and it has been suggested that the miRs are potentially involved in the pathogenesis of solid organ rejection, including renal, intestinal and hepatic rejection. However the role of miRs on lung IRI has not been completely understood yet. Lidocaine (lido), a commonly used local anesthetic agent, has proved its anti-inflammatory activity in several tissues including lung but its possible modulation of miRs has not been investigated.
Aim: To investigate a potential involvement of miRNAs in lung IRI in a lung auto-transplant model. In addition the effect of lidocaine was investigated.
Animals and Methods: 3 groups (Sham-operated, control and lido) of 6 large-white pigs each were submitted to a left lung auto-transplant. All groups received the same anesthesia. In addition animals of lidocaine group received a continuous IV administration of lidocaine (1.5 mg/Kg/h) during surgery. In order to measure the expression of miRs, lung tissue samples were taken at: 1) 5 minutes before pulmonary artery clamp (PPn), 2) 5 min before reperfusion (PRp), 3) 30 min post-reperfusion (PR30) and 4) 60 min post-reperfusion (PR60). Lung tissue samples were analyzed for miRs (miR-122, miR-145, miR-146a, miR-182, miR-107, miR-192, miR-16, miR-21, miR-126, miR-127, miR142-5p, miR152, miR155, miR-223 and let7) using RT-QPCR. Results were normalized using miR-103.
Results: The expression of miR-127 and mir-16 did not increase after IRI. All the other miRs investigated exhibited more than two-fold differences at the PR60 time point and this effect was positively correlated with the severity of IRI. This increase in miRs levels was significantly down-regulated by lido administration.
Conclusions: Our results support the notion that IRI causes changes in miRs expression that can be used as markers of injury. In addition, lidocaine administration modifies miRs expression.
Keywords: ischemia reperfusion injury, lung, miRNAs.

MON-052
MicroRNA expression profiling predicts melanoma metastatic potential

A. V. Kudryavtseva[1,2], A. A. Dmitriev[1,2], A. V. Snezhkina[1], A. F. Sadritdinova[1,2], N. V. Melnikova[1], V. A. Lakunina[1], L. A. Uroshlev[1], A. A. Rogozhnikova[1], G. A. Petukhova[2], E. N. Slavnova[2], B. Y. Alekseev[2], N. N. Volchenko[2], A. S. Zasedatelev[1,3]
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Melanoma is aggressive skin, uveal or mucosal tumor with early metastatic onset. Even small tumors are capable of producing distant metastases. Uncertainties in the detection of metastases complicate the choice of a suitable treatment strategy.

The aim of our work is to develop a set of microRNA capable of discrimination between non-invasive and metastatic melanoma. We performed miRNA-seq analysis of 14 benign skin tumors, 18 primary melanoma neoplasms including 7 metastatic ones. We identified the set of 23 microRNA with statistically significant expression alterations between metastatic and non-metastatic melanomas ($p < 0.05$) and 17 microRNA – between benign tumors and malignant melanomas. Additionally we performed a screening of literature results obtained previously by other groups and finally collected a set of 31 microRNA with presumably differential expression patterns between metastatic and non-invasive melanomas, and a set of 25 microRNA – between melanomas and benign tumors. RT-qPCR validation allowed to identify a subset of miR-145, miR-150, miR-155, miR-193a/b, miR-196a, miR-211, miR-214, miR-221/222, miR-342-3p, miR-455-3p and miR-497 capable of discrimination between melanoma skin cancer and benign tumors (with overall sensitivity of approx. 90% and specificity of 85%), and subset of miR-30b miR-145, miR-149, miR-155, miR-182, miR-200a/b/c, miR-221/222 and miR-497 for the evaluation of the presence of metastases (sensitivity 87% and specificity 79%). These biomarker sets includes well-known miR-182 targeting *FOXO3* and miR-30b targeting *GALNT7*.

Further validation of these microRNA biomarker candidates on an extended cohort of patients would allow the creation of test-systems capable of detecting both malignant melanomas and discriminate tumors with high metastatic potential to facilitate the choice of appropriate therapy strategy.

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Keywords: melanoma, metastases, microRNA.

8. **L Rancan**, K Aymmonier, S.D. Paredes, E Marchal-Duval, J Casanova, I Garutti, C Simón, E Vara. Protective effect of lidocaine against glycocalix damage secondary to lung ischemia reperfusion. The FEBS EMBO 2014 Conference, Paris, France. 30 Agosto – 4 Septiembre 2014

SUN-269
Protective effect of lidocaine against glycocalyx damage secondary to lung ischemia reperfusion

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Background: Increased vascular permeability is a characteristic feature of ischemia reperfusion injury (IRI) that has been ascribed to a malfunction of endothelial cells. Recently it has become evident that the endothelial glycocalyx (EGX) is of considerable importance concerning various aspects of vascular physiology. It is involved in inflammatory and immune reactions and it mediates the release of vascular regulatory agents such as nitric oxide. Another important biological property of the EGX is the ability to create a barrier between the endothelium and the blood cells to prevent cell adhesion. The degradation of the EGX is associated with increased circulating levels of the major EGX components heparan sulfate (HS) and syndecan-1 (Synd-1). Lidocaine is a commonly used local anesthetic agent which has also been found to possess anti-inflammatory activity in several tissues including lung. However, the influence of lidocaine on glycocalyx structure has not been investigated.
Aim: To investigate a possible protective effect of lidocaine on lung glycocalyx injury secondary to IRI.
Animals and Methods: 2 groups (control and lidocaine) of 6 large-white pigs were submitted to left lung auto-transplant. Both groups received the same anesthetic induction (fentanyl 3 µg/kg, propofol 3 mg/kg, atracurium 0.5 mg/kg). In addition animals of lidocaine group received a continuous IV of lidocaine 1.5 mg/kg/h during surgery. Blood samples were taken in four different moments, 1) pre-pneumonectomy (PPn) (5 min before pulmonary artery clamp), 2) pre-reperfusion (PRp) (5 min before reperfusion), 3) 30 min post-reperfusion (PR30) and 4) 60 min post-reperfusion (PR60), in order to measure the levels of glycocalyx markers Synd-1, HS and Cathepsin B. Intercellular adhesion molecule-1 (ICAM-1) was also measured.
Results: Levels of Synd-1 were markedly higher ($p < 0.05$) at PR30 compared to PPn and PRp values. The increase was even higher at PR60. IRI also increased HS and ICAM-1 concentrations in plasma ($p < 0.05$). On the contrary, decreased syndecan-1 levels were observed in the lung ($p < 0.05$) after reperfusion. All these effects were partially blocked by lidocaine.
Conclusions: Our findings demonstrate the contribution of the endothelial glycocalyx to the lung IRI. Lidocaine prevented the deleterious effect on glycocalyx markers suggesting that lidocaine administration may aid to protect the glycocalyx.
Keywords: glycocalyx, ischemia reperfusion injury, lung.

ation of neurons. Numerous empirical evidences have supported the use of LA as anti-inflammatory nutraceutical since it evokes an exclusive array of cellular and molecular mechanisms, albeit its conclusive molecular mechanisms in Parkinson's disease are still not completely understood. We investigated the neuroprotective effects of (R)-(+)-α-Lipoic Acid(R-LA) on 1-methyl-4-phenylpyridinium (MPP^+)-induced activation of microglia BV-2 and neurotoxicity on dopaminergic SH-SY5Y cells. We then investigated the protective effects of R-LA in co-cultured BV-2 and dopaminergic SH-SY5Y cells stimulated with MPP^+. R-LA mitigated MPP^+-induced cell death in both dopaminergic SH-SY5Y and activated BV-2 cells. Pretreatment with R-LA significantly attenuated MPP^+-induced overexpression of inducible nitric oxide synthase (iNOS) and subsequent production of nitric oxide in BV-2 cells. R-LA treatment induced the activation of PI3K-Akt which then reduced the cyclooxygenase (COX)-2 expression, inactivated GSK-3β(Ser9) and supressed the p65NF-κβ translocation in BV-2 cells. Following this, R-LA aggrandized the level of anti-inflammatory cytokine IL-10 which concomitant diminution of pro-inflammatory cytokines TNF-α, IL-Iβ, IL-2 and IL-6. Furthermore, R-LA independently decreased MPP^+-induced oxidative stress in SH-SY5Y by reducing intracellular ROS level, aggrandizing the GSH level and the expression of heme oxygenase-1. Following co-culture of BV-2 and SH-SY5Y cells, neuronal cell death was inhibited where nuclear condensation and apoptotic bodies was abated with intact mitochondrial membrane potential which led to the suppression of caspase-dependent apoptosis as compared to MPP^+-treated co-cultured cells. Moreover, the addition of lithium chloride and triciribine hydrate (API-2) resulted in the prolonged BV-2 activation, elevation of pro-inflammatory cytokines which led to neuronal cell death in SH-SY5Y cells. This further confirmed R-LA protected the BV-2 and dopaminergic SH-SY5Y against MPP^+ through PI3K-Akt/GSK-3β pathway. In conclusion, neuroprotective effects of R-LA against MPP^+ are mediated, at least in part, through suppression of neuroinflammation and oxidative stress-associated factors in BV-2 cells. This further justifies the rational use of R-LA as nutraceutical for neurodegenerative diseases.
Keywords: neurodegenerative diseases, Neuroinflammation, Neuronal cell death.

SUN-271
Protective effects of 2-benzoxazolinone derivatives with anti-oxidant and anti-inflammatory activities on experimental acute pancreatitis in rats

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9. **L. Rancan**, S.D. Paredes, C. García, C. Tello, P. Guillen, I. Garutti, C. Simón, E. Vara. MicroRNAs as biomarkers in lung transplantation. 50th Golden Anniversary Congress of the European Society for Surgical Research, Liverpool , UK 10-13 Junio 2015

MicroRNAs as biomarkers in lung transplantation

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Objective: Lungs are characterized by the highest rates of rejection among the transplanted solid organs. Thus, there is a critical need for biomarkers that serve to establish donor organ quality, early diagnosis of graft injury and treatment response. MicroRNAs (miRNAs) have emerged as promising disease biomarkers. The aim of this study was to examine the possibility that changes in miRNA expression may be used as biomarkers for ischemia-reperfusion injury (IRI) in a model of lung autotransplantation. A possible correlation with changes in glycocalyx and the effect of lidocaine were also investigated.
Methods: Three groups (sham-operated, control and lidocaine) of 6 large-white pigs each were submitted to a left cranial lobe-lung autotransplantation. Lung tissue samples were taken at: 1) 5 min before pulmonary artery clamp (PPn), 2) 5 min before reperfusion (PRp), 3) 30 min post-reperfusion (Rp-30) and 4) 60 min post-reperfusion (Rp-60) and analyzed for miRNA patterns.
Results: miR-145, miR-146a, miR-182, miR-10, miR-192, miR-21, miR-126, miR142-5p, miR152, miR155, miR-223 and let7 were differentially expressed in the control group compared to the expression observed in the sham group ($p<0.05$). Interestingly, we identified glycocalyx markers syndecan-1 and heparan sulfate as miRNA target candidates for miR126 and miR142-5p. IR also increased syndecan-1 and heparan sulfate concentrations in plasma ($p<0.05$) and decreased syndecan-1 levels in lung ($p<0.05$). These effects were partially prevented by lidocaine.
Conclusion: IRI produced changes in miRNA expression. Moreover, we found a distinctive miRNA expression pattern in lung tissue in response to lidocaine.
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Congresos como coautor

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8. D. Rincón, **L. Rancan,** G. Sánchez-Pedrosa, F.J. González-Moraga, P. Manu, I. Garutti, C. Simón, E. Vara Effect of sevoflurane on pulmonary glycocalyx alteration in response to one-lung ventilation. 50th Golden Anniversary Congress of the European Society for Surgical Research, Liverpool , UK 10-13 Junio 2015

Otros méritos

- Premios Ulysses de investigación 2012: Primer premio en la categoría “Premio al desarrollo complementario de la formación médica”.
- Premio Walter Brendel Award 2012 (European Society for Surgical Research): premio a la investigación experimental para el trabajo “Sevoflurane prevents liver inflammatory response indued by lung ischaemia / reperfusion in a lung autotransplant model
- Mejor abstract sobre trasplante al 49 Congreso de la Sociedad Europea de Investigación quirúrgica por parte de la Sociedad Europea de Trasplante – Comitee de jovenes profesionales sobre Trasplante 21-24 mayo 2014